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American Journal of Veterinary RESEARCH

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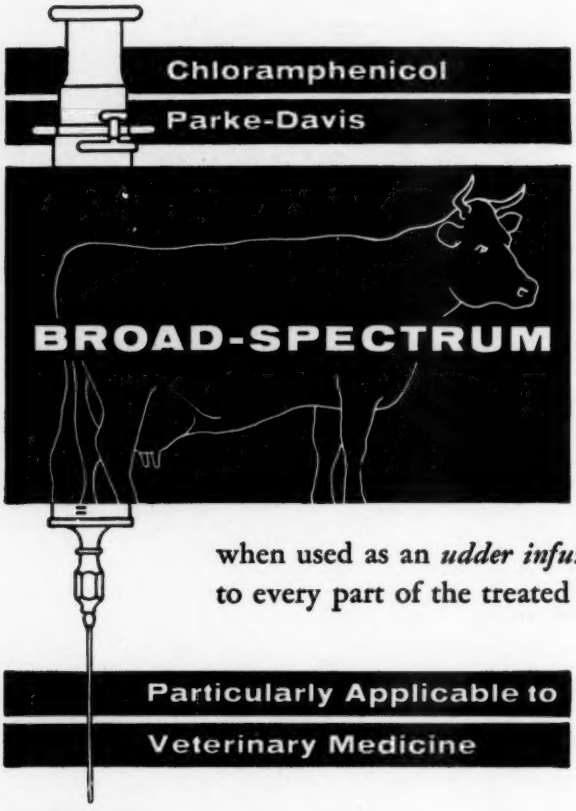

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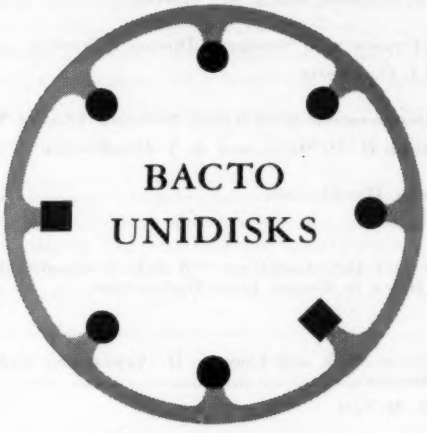

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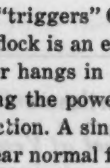

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White Muscle Disease (Myopathy) in Lambs and Calves. VI. Effects of Selenium and Vitamin E on Lambs

O. H. MUTH, D.V.M.; J. E. OLDFIELD, Ph.D.; J. R. SCHUBERT, Ph.D.; L. F. REMMERT, Ph.D.

Corvallis, Oregon

WHITE MUSCLE DISEASE (WMD) of cattle and sheep has eluded practical solution since it was recognized as a distinct entity over a quarter of a century ago.[6, 12]

Because the characterizing lesions[12, 18, 26] simulate those of naturally occurring[4, 6] and experimentally induced[1, 4, 21] avitaminosis E in these species, some have concluded that WMD is an uncomplicated vitamin deficiency, and the expression, "white muscle disease," has been used to denote any grossly apparent myopathy. We prefer to use that expression when referring to a specific myopthy of lambs and calves resulting when the dams have been fed during gestation on feeds, especially legumes, grown in certain areas. It seems reasonable to limit the use of the expression in this manner until a more suitable nomenclature is suggested. Experimental feeding to cows and ewes of alfalfa and clover hays grown in certain areas has resulted in a high incidence of WMD in their offspring,[16, 17, 31] and this has been a great aid in studying the disease.

Although there have been some reports of the successful use of vitamin E in WMD,[10, 11, 20, 22, 30] studies of blood tocopherol levels,[15, 20, 22] feed tocopherol levels,[5, 9, 15, 20, 22, 25, 30] and the supplementation of the dams' rations with vitamin E[13] have tended to refute the hypothesis that WMD results from an uncomplicated vitamin E deficiency.

The inclusion of selenium in a natural WMD-genic ration was prompted by recent reports of Schwarz and Foltz[25] relative to the protection afforded by selenium against "dietary hepatic necrosis" in rats, of DeWitt and Schwarz[5] relative to "multiple dietary necrotic degeneration" in mice, and of Patterson *et al.*[18] and Schwarz *et al.*[24] relative to the protection afforded by selenium against "exudative diathesis" in chicks. Consequently, trials were designed and executed wherein the effect of selenium and vitamin E administered to pregnant ewes could be evaluated.

A brief technical report of this work has been published.[14] This paper is a more detailed report of the experiment and discusses the results and implications. Blood chemical studies will be reported in the succeeding paper.

MATERIALS AND METHODS

Mature ewes of mixed breed were maintained during the summer months on mixed pasture in an area where WMD is uncommon. They were randomized into five lots of 12 each and housed in a shed open to the weather on one side and provided with outside exercise runs. Inside and outside areas were littered with wood shavings that were renewed frequently. Fresh water and iodized block salt were provided ad libitum.

The control ration fed in lot 1 consisted of 2.0 lb. of alfalfa and 2.0 lb. of Ladino clover hays grown in an area where WMD is not a serious problem, plus 0.25 lb. of oats per ewe per day. This batch of oats had proved to have no protective effect in trials conducted the previous year.[17] The basal experimental ration fed to each ewe in lots 2, 3, 4, and 5 consisted of 4.0 lb. of Ladino clover hay plus 0.25 lb. of oats. This hay originated from an irrigated area where the clover is grown for seed and was the first cutting (clipping) removed to stimulate flower production of the plant. It graded "U.S. No. 1 green leafy" and had an *alpha*-tocopherol content of about 25 μg. per gram[27] and a selenium content of less than

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The assistance of Mr. Phillip E. Williams in the care of the experimental animals is acknowledged.

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0.1 p.p.m., the limit of the analytical method employed.[7] Hay originating on the same ranch proved capable of eliciting the disease in a high percentage of lambs when experimentally fed to their dams the previous year.[17]

In addition to the basal experimental ration, each ewe in lot 3 was injected intramuscularly each week with 770 I.U. of vitamin E as d-*alpha*-tocopheryl polyethylene glycol 1,000 succinate.* Ewes in lot 4 were each fed 100 I.U. of vitamin E per day in the oats as d-*alpha*-tocopheryl acetate (Myvamix*), and those in lot 5 were fed 0.1 p.p.m. of selenium (calculated for the entire ration) in the oats as sodium selenite. A preliminary trial indicated that the stated dosage of d-*alpha*-tocopheryl polyethylene glycol 1,000 succinate was sufficient to maintain satisfactory blood levels in ewes on clover hay.

Rams were placed with the ewes on September 11. Fifty days later the ewes were started on their respective experimental regimes, which were continued for 140 days. The first lamb was born 94 days after the ewes were started on experimental feeding and all lambs were born within the 140-day regimes.

One of each pair of twins born in lots 1 and 2 was killed within the first three days after birth. Surviving lambs were killed at approximately 6 weeks of age. All lambs were necropsied and tissues were selected for histopathological and chemical study.

Blocks of tissue were routinely taken from the left portion of the heart below the coronary groove, including a portion of the ventricular septum, as well as portions of the adjacent right and left ventricular walls. Blocks also were routinely taken from the proximal portion of the semitendinosus muscle. Additional blocks were taken from other sites when indicated. Tissues were fixed in neutral buffered formalin solution, processed, cut at 6 μ, stained with hematoxylin and eosin and with Von Kossa's silver nitrate to demonstrate calcium.

Diagnosis of WMD was made on recognition of characteristic gross or microscopic lesions, or both.

RESULTS

The number of lambs in each group which developed lesions or signs of WMD and the rations fed to their dams are shown (table 1). The lambs in the groups varied in number from 15 to 20.

The 1 affected lamb in lot 1 was killed at 50 days of age. One 3- by 4-mm. subendocardial plaque was observed on the intraventricular septum. The lamb appeared normal otherwise. The 4 nonaffected lambs (1 of each pair of twins) in lot 2 were examined when less than 1 week of age. In our experience, WMD does occur at this age, but it is uncommon. The 4 nonaffected lambs in lot 3 were examined at 2, 3, 3, and 47 days of age, respectively. The 4 nonaffected lambs in lot 4 were examined at 1, 2, 41, and 49 days of age, the last 2 being twins. In the case of lot 5, the last 3 lambs born were found to have only microscopic lesions. Of these, 1 was examined at 47 days of age and 22 days after terminating selenium feeding. The other 2 (twins) were examined at 56 days of age, 34 days after selenium feeding had ceased. When killed, these lambs were fat and clinically healthy. When these facts are considered, the results of the trials appear more significant than is indicated (table 1).

TABLE 1—The Rations Fed to Each Group of 12 Ewes and the Number of Their Lambs Which Developed White Muscle Disease

Lot	Ration	Results	
		Affected	Nonaffected
1	Control	1(0)	17
2	Basal experimental	11(5)	4
3	Basal experimental plus parenteral vitamin E	11(3)	4
4	Basal experimental plus oral vitamin E	16(7)	4
5	Basal experimental plus oral sodium selenite	3(0)	13

The figures in parentheses indicate numbers of lambs showing clinical signs of stiffness.

DISCUSSION

It appears from these results that under the conditions of this experiment, a minute amount of selenium had a strong protective action against WMD. In view of the other reports concerning the dietary effect of selenium,[5, 18, 24, 25] this should not be surprising. This is especially true in the case of the "multiple necrotic degeneration" reported by DeWitt and Schwarz[5] which, in Schwarz's[23] opinion, is similar to the WMD described by Muth.[13] Although, in the past, the interest in selenium on the part of animal nutritionists and veterinarians has been largely because of its toxic properties, which have constituted an economic problem for the livestock industry in several states, this interest did lead to much fundamental research concerning that element and its biological properties. It is of interest that Poley *et al.*[19] reported that subtoxic levels of selenium produced a positive growth response in chicks, a phenomenon that has waited 16 years for exploitation.

Trelease and Beath[29] state that sulfate

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in the soil inhibits the uptake of selenite, which may explain the frequent "storms" of WMD following fertilization with the common sulfur-bearing fertilizers.[13] Livestock men in central Oregon have repeatedly reported severe losses from this disease following the use of elemental sulfur alone as a fertilizer in areas where forage yield response is marked following its use.

The finding of lesions in 3 lambs in lot 5 on days 22 and 34 after feeding of selenium to the dams had ceased seems to indicate that they may become depleted during that period, and that perhaps a higher level of intake than was used in this experiment might be practical.

One can only postulate concerning the true role of selenium in the body processes. It is generally recognized that materials entering into biological processes, in the amounts indicated by the various reports concerning selenium's role as an essential element, enter into enzyme systems. The extremely small amount of selenium, when supplied as factor 3,[25] required to fulfill the apparent essential requirements is striking in this respect.

It also appears from these results that vitamin E administered both orally and parenterally to the ewes had little or no effect on the occurrence of WMD in the lambs. This seems to indicate that the rations already contained sufficient vitamin E. While U.S. No. 1 green leafy clover hay was used as the principal ingredient of the experimental ration, it should be pointed out that the quality of that feed was not significantly different from many other leguminous hays commonly fed in the northwest intermountain area and, although the vitamin E requirements for sheep are unknown, the hay fed probably did furnish sufficient amounts of that vitamin.

Previous experience[17] had demonstrated that relying on clinical signs such as "stiffness" as criteria of myopathy is a fallacy, especially in the case of heart lesions, and to a lesser extent in the case of skeletal muscle lesions.

This was again demonstrated in this experiment, as illustrated by the relatively few lambs showing clinical signs (table 1). If these sheep, which were rather closely confined, had been turned out and subjected to more strenuous exercise, a larger number of clinical cases of the disease might have been observed.[12] No arthritis, which is a common cause of "stiffness" in lambs, occurred in this experiment.

Nine of the lambs having well-defined microscopic lesions would not have been recognized as affected if their tissues had not been examined microscopically. This accounts for the discrepancy in the numbers given in the preliminary report,[14] which was based on gross examination. This shows the difficulty that may be encountered in attempting to evaluate clinical diagnosis and treatment of affected animals and emphasizes the necessity of good design and execution of experiments for this purpose.

Although it has been shown in the rat[25] and chick[18,24] that cystine, vitamin E, and selenium are somewhat interchangeable in preventing a deficiency disease, the results of this experiment indicate that this is not necessarily true in the case of sheep, since the administration of additional vitamin E to the ewes in lots 3 and 4 did not materially alter the incidence of WMD in the lambs.

In view of the accumulating evidence concerning selenium as an essential element in the nutrition of rats,[25] chicks,[18,24] mice,[5] and sheep, the statement of Schwarz and Foltz[25] to that effect appears reasonable. This indicates the need for further critical investigations relative to this element in other myopathies occurring in animals and man.

SUMMARY AND CONCLUSIONS

Five lots of 12 pregnant ewes each were fed as follows: (1) a normal control ration; (2) a basal experimental white muscle disease (WMD)-genic ration; (3) the basal experimental ration plus vitamin E parenterally; (4) the basal experimental ration plus oral vitamin E; and (5) the basal experimental ration plus oral sodium selenite. In the lambs born to these ewes, there was a high incidence of WMD, except in the normal control and selenium supplemented rations.

It seems evident that, under the conditions of this experiment, sodium selenite, when fed to supply 0.1 p.p.m. of selenium in the ration, exerted a strong protective effect against WMD.

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A Succinoxidase Inhibitor in Feeds Associated with Muscular Dystrophy in Lambs and Calves

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A NATURALLY OCCURRING muscular dystrophy in lambs and calves in certain areas of Montana and other states has been shown to be associated neither with low levels of vitamin E in the ration, nor with low blood, colostrum, or milk tocopherol.[11, 12, 17] The disease occurs in lambs and calves born of mothers which have been fed hay grown and cured in the affected areas.[6-9, 11, 16, 18] The cause of the disease may lie in a nutritional deficiency in the feed, or it may be due to the presence of a toxic agent.

On the theory that the initial metabolic defect in the dystrophic muscles may lie in the aerobic phase of muscle metabolism, it was decided to determine whether any substance in the dystrophogenic feeds interferes with the cytochrome oxidative chain, as measured by the complete succinoxidase system. For this purpose, extracts of dystrophogenic and nondystrophogenic feeds were added to the succinoxidase system for the detection of any inhibitory materials. This paper reports the discovery of such an inhibitor in feeds associated with muscular dystrophy outbreaks, together with data on the formation of the inhibitor during the curing of the hay, and evidence for the partial reversal of the inhibition by the addition of tocopherol to the system.

MATERIALS AND METHODS

Feeds Tested.—Feed samples were obtained from Oregon,* New York,† and Montana. In some cases, the feeds were secured from ranches with known histories of muscular dystrophy or from ranches free of the disease. In other cases, the feeds were those used in the experimental production of muscular dystrophy. In each case, it was possible to describe the feed as probably dystrophogenic or probably nondystrophogenic. In Montana, samples were obtained by cutting cores from several places in the hay stacks with a wool coring machine. Several cores were combined, ground, and reduced to samples of suitable size. The manner of obtaining the Oregon and New York samples was not known. Samples from growing hay crops, or from hay cut but still in the swath, were obtained by hand picking a number of small bunches of hay, which were then dried quickly on elevated wooden platforms in the sun and breeze, and ground.

Extraction.—Varieties of solvents were tested for their effectiveness in extracting the inhibitor. Hot ethanol was finally selected as the most suitable. Ten-gram samples of the ground feeds were extracted exhaustively in Soxhlet extractors with ethanol. The ethanol extracts were concentrated to 10.0 ml., so that 0.001 ml. of extract contained the activity from 1.0 mg. of the feed.

Measurement of Inhibition.—The succinoxidase system was that described by Schneider and Potter.[14] Each Warburg flask contained 1.0 ml. of phosphate buffer, 0.4 ml. of 10^{-4} M cytochrome c, 0.3 ml. of 0.004 M calcium chloride, 0.3 ml. of 0.004 M aluminum chloride, 0.3 ml. of 0.5 M sodium succinate, 0.2 ml. of mouse liver homogenate containing 30.0 mg. liver (wet weight), 0.3 ml. of 2.0 M sodium hydroxide in the center well, and 0.1 ml. of an ethanol solution of the inhibitor diluted to contain the extract from 80 mg. of feed (less for high potency extracts). In all cases, 0.1 ml. of ethanol was added to the controls to correct the slight inhibition due to the ethanol itself. The gas phase was air. In the early experiments the succinate was tipped from a side arm to initiate the reaction after equilibration, but in later runs it was found more satisfactory to tip the liver homogenate from the side arm. Readings were taken at ten-minute intervals for at least an hour. Because of slight variations from one mouse liver to the next, the extract of a single hay, known to be dystrophogenic, was used as a standard inhibitor, and all others were compared with it. The unit was the inhibitory activity of 0.001 ml. of this extract, *i.e.*, the activity from 1.0 mg. of this particular hay.

RESULTS

Relationship of Succinoxidase Inhibitor to Muscular Dystrophy.—The succinoxidase inhibitor (SI) levels in the various samples are presented (table 1). It is evident that high values (above 550 units) were more commonly found on ranches with histories of muscular dystrophy than on ranches without muscular dystrophy.

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* Supplied by Dr. O. H. Muth, Oregon State College.
† Supplied by Dr. D. E. Hogue, Cornell University.

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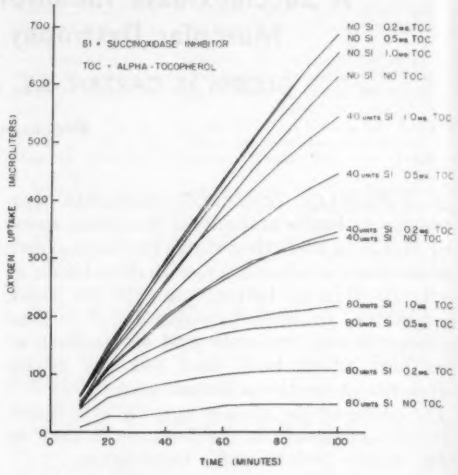
Eleven of the 15 ranches with muscular dystrophy had samples with high SI levels, while only three of the nondystrophy ranches had high levels.

The Formation of Succinoxidase Inhibitor in Hay During the Curing Process.—

One field of mixed alfalfa and alsike clover located in an affected area was sampled at intervals immediately preceding and during the curing process. The results of the SI assays are given (table 2). It is evident that the growing crop contained very little SI, but that it appeared during the curing process, especially if the curing was delayed by rain.

Partial Reversal of the Succinoxidase Inhibitor Effect by Tocopherol.—There is evidence [13] that the addition of high levels of d-*alpha*-tocopherol acetate to the dystrophy-producing hay ration of ewes partially prevented the occurrence of muscular dystrophy in their lambs. Moreover, injection of a water-soluble preparation of tocopherol into dystrophic lambs resulted in recovery of about half of these that otherwise would have died. It was of interest, therefore, to determine whether *alpha*-tocopherol added to the respiring succinoxidase system would counteract the inhibition caused by the SI factor of dystrophogenic hay. In the experiment illustrated (graph 1), the tocopherol and SI were added simultaneously to the Warburg flasks containing all the reactants except succinate, which was tipped from the side arm after equilibration. Other experiments had shown that neither the order of addition of the inhibitor and the tocopherol nor the time that the two were in contact with each other before their addition influenced the course of their effect. It may be seen (graph 1) that tocopherol had almost no effect on the uninhibited system, but when the SI was present in the system, tocopherol partially reversed its inhibitory effect, and the reversal was greater with increasing amounts of tocopherol.



Graph 1—Partial reversal of the succinoxidase inhibitor effect by tocopherol.

TABLE 1—Relative Inhibitions of the Succinoxidase System by Feed Extracts

Ranch No.	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
	NONDYSTROPHOGENIC RANCHES				
1	370*	.....	.....	.....	.....
2	380	.....	.....	.....	.....
3	450	.....	.....	.....	.....
4	−20	940†	120	300	.....
5	320	.....	.....	.....	.....
6	280	.....	.....	.....	.....
7	330	360	.....	.....	.....
8	230	.....	.....	.....	.....
9	530	.....	.....	.....	.....
10	440	430	280	290	.....
11	220	860	.....	.....	.....
12 (silage)	980	.....	.....	.....	.....
	DYSTROPHOGENIC RANCHES				
13	390	1,000**	660	870	.....
14	510	160	660	−190	140
15	950	.....	.....	.....	.....
16	140	230	.....	.....	.....
17	910	.....	.....	.....	.....
18	1,000	310	770	.....	.....
19	550	.....	.....	.....	.....
20	720	210	.....	.....	.....
21	440	.....	.....	.....	.....
22	830	.....	.....	.....	.....
23	200	.....	.....	.....	.....
24	110	.....	.....	.....	.....
25	670	.....	.....	.....	.....
26	920	.....	.....	.....	.....
27	660	510	.....	.....	.....

* All values expressed as inhibitory units per gram of feed. † Underscored numbers indicate values above 550 units per gram. ** This was the standard extract against which all others were compared.

TABLE 2—Formation of Succinoxidase Inhibitor in Hay During the Curing Process

Date of Sampling	Stage	Succinoxidase inhibitor (units)
6/24/57	Growing	110
7/3/57	New mown	70
7/5/57	In swath 2 days, rained on twice	360
10/14/57	From stored bales	660
10/14/57	From bales repeatedly dampened before stacking	>1,000

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Effect of Succinoxidase Inhibitor on Other Enzyme Systems.—To eliminate the possibility that the SI effect was a nonspecific inhibition of enzyme action, its effects on the xanthine oxidase system, the cytochrome oxidase system, and the succinic dehydrogenase system were measured.

Xanthine oxidase activity, expressed as microliters of oxygen uptake by 167 mg. mouse liver in 130 minutes at 37 C., using hypoxanthine as substrate,[10] was 42 without SI, 40 with 50 units of SI, and 47 with 100 units of SI. Thus, there was no inhibition.

Cytochrome oxidase activity was measured using ascorbate as an electron donor and liver homogenate as an enzyme source.[14] In the absence of SI, the activity of 2 mg. of liver was 146 μl. O_2 in 60 minutes; with 50 units of SI it was 56 μl.; and with 100 units of SI it was 49 μl., so there was a distinct inhibition of this system, though this inhibition was not appreciably greater at the higher level of SI. The 100-unit level of SI would have totally inhibited a complete succinoxidase system respiring five times as rapidly, so actually the inhibition of the cytochrome oxidase system was relatively small as compared with the inhibition of the succinoxidase system.

Inhibition of the succinic dehydrogenase system, using methylene blue as electron acceptor, was measured only qualitatively. It was found that SI prevented the reduction of methylene blue by liver homogenate in the presence of succinate.

Discussion

These observations are of a preliminary nature and leave much to be done on the purification and characterization of SI and the determination of its exact mode of action, as well as the nature of the tocopherol antagonism. It would be premature at this point to discuss these matters.

To be considered here is whether this evidence is sufficient to support a hypothesis that the muscular dystrophy-producing property of certain feeds is due to the presence of the SI factor in these feeds.

In considering the etiological significance of SI in naturally occurring muscular dystrophy, it must be kept in mind that a vitamin E deficiency might also be involved in some cases.[2] For example, the feed from ranch 23 (table 1) was associated with a high incidence of muscular dystrophy, though it contained only 200 units of SI per gram, but this was a sample of Ladino straw, a type of feed expected to be low in tocopherol.

The difficulty in obtaining a proper sampling of feeds stored on cattle and sheep ranches also may lead to some discrepancies. If the sample brought to the laboratory is not representative of the feed being provided to the pregnant cows or ewes during the critical period, there may be a misinterpretation of the data. However, in this study almost every ranch with a history of muscular dystrophy had at least one sample with a high level of SI. On the other hand, only three of the 12 nondystrophy ranches had samples with high levels of SI. One of these three ranches (ranch 4) provided four samples with only one of them showing high SI. A second ranch (ranch 11) had samples with one low and one high SI level; these two hay samples were first and second cuttings from the same field. Only one sample was submitted from the third ranch (ranch 12) and, this being a silage sample, may have contained succinoxidase inhibitors chemically and physiologically different from the SI factor found in the other feeds, which were all dry roughages.

A variety of substances, such as unsaturated fatty acids,[3] plasmocid,[5] tri-*o*-cresyl phosphate,[4] diphtheria toxin,[15] and potassium iodide,[1] are known to induce muscular dystrophy. It is conceivable that a naturally occurring substance might have the same effect, and the data presented here appear to support the theory that SI is such a substance.

Whether SI is the result of an autolytic process or the product of the action of saprophytes on the moist hay is uncertain. The SI is not antimycin, the cytochrome inhibitor elaborated by Streptomyces, as evidenced by differing solubility behavior and differing reaction kinetics on the succinoxidase system. However, it might be another product of bacterial or fungal activity, since it is formed under conditions conducive to such activity, although the affected hay is ordinarily only slightly discolored and not visibly moldy.

Supplementary feeds have been discovered which have an unknown ingredient capable of counteracting the dystrophy-producing properties of hays high in SI.[12] Very high levels of vitamin E in the ewes' rations also had this effect to a limited

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extent.[18] The failure of a feed that is high in SI to produce muscular dystrophy may indicate the presence of either of these protective materials. Chemical analysis for vitamin E is possible, but detection of the other protective agent is achieved only through ewe feeding trials which involve large amounts of the feed being tested and many months of time.

The *in vitro* antagonistic action of *alpha*-tocopherol (vitamin E) and the SI factor in the succinoxidase system, as well as a similar antagonism between tocopherol and dystrophogenic hay *in vivo*, suggest that the SI factor may be etiologically important in naturally occurring muscular dystrophy.

Final establishment of the significance of SI must eventually involve improved sampling methods, adequate control of vitamin E and other protective factor levels in the rations, a study of the metabolism of SI, and the production of muscular dystrophy by feeding purified SI to experimental animals.

SUMMARY

1) An ethanol-soluble factor found in hay and other feeds was inhibitory to the succinoxidase system.

2) This factor was more abundant in feeds associated with muscular dystrophy in calves and lambs than in feeds with no such association, though there were some exceptions to this trend.

3) The factor was almost absent from growing hay but appeared during the curing process, especially if curing was prolonged by rain and cool weather.

4) The succinoxidase inhibitory activity of this factor was partly reversed by the addition of *alpha*-tocopherol to the system.

5) The succinic dehydrogenase system was inhibited, the cytochrome oxidase system was partly inhibited, and the xanthine oxidase system was not inhibited by levels of the factor which completely inhibited the succinoxidase system.

6) An hypothesis is suggested to the effect that the succinoxidase inhibitor is responsible for muscular dystrophy in lambs and calves born of dams maintained on feeds containing excessive amounts of this inhibitor. The evidence was indicative but not conclusive.

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Procurement of Lambs by Hysterectomy and Their Isolation

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RESEARCH in the infectious diseases of large animals has been seriously hampered by an inadequate supply of suitable experimental animals. Subclinical enzootic infections in herds and flocks or their environment result in a complicated antibody background and makes the animal of uncertain value for experimental purposes.

Animals with known immunological backgrounds are essential in the investigation of diseases whose distribution, etiology and transmission are unknown. Techniques for measuring susceptibility in individuals without actual exposure to the etiological agent have not been developed. It is, therefore, impossible to screen large numbers of animals to secure susceptible ones.

Colostrum-deprived, antibody-devoid pigs, secured by the hysterectomy technique described by Young *et al.*[7] or by hysterotomy as used by Hoerlein *et al.*,[1] provide researchers in swine diseases with uniformly susceptible animals.

Certain diseases, such as shipping fever and listeriosis, are known to occur in both cattle and sheep. This indicates that sheep which are free from environmental exposure can be satisfactorily used for the investigation of diseases of cattle as well as of sheep. Lambs obtained by the hysterectomy technique would be colostrum-deprived and so should be antibody-devoid. Use of aseptic isolation units and procedures as described by Young and Underdahl[6] would protect the lambs from environmental factors and maintain their susceptibility.

This is a report of the application of these hysterectomy and isolation techniques to procure lambs for experimental disease studies.

MATERIALS AND METHODS

Breeding Stock.—The 11 ewes used in this preliminary study, 9 aged Western ewes and 2 long yearling Corriedales, were handmated with Corriedale rams during September to December. A known breeding date permitted the scheduling of the hysterectomy three to eight days prior to expected parturition. The ewes were sheared approximately one month before hystrectomy.

Equipment.—The hysterectomy hood (fig. 1) used for pigs by Underdahl and Young[4] was employed. A steel drum, large enough to admit the head and shoulders of the ewe, was used for administration of the CO_2 anesthetic.

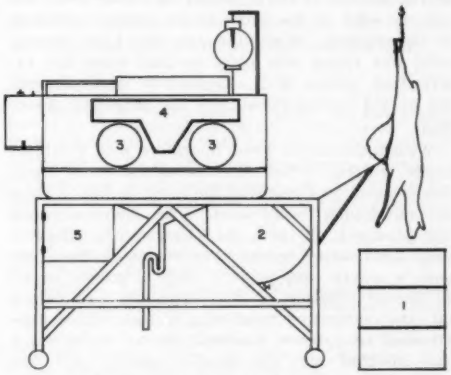

Fig. 1—Schematic drawing of the hysterectomy hood showing (1) steel drum for CO_2, (2) water lock, (3) holes for operator's arms, (4) window in sterile hood, and (5) door for removal of lambs.

An individual isolation unit* constructed from fiberglass (fig. 2) was provided for each lamb and was principally the same as those used for pigs. The unit (26 by 26 by 27 inches) provided ample space for the lamb during several months of isolation. The intake and exhaust air was filtered through glass wool pads† and drawn through the unit by a squirrel-cage fan.

A small door, 3 inches above the expanded metal floor, permitted the introduction of the milk pan. The lamb could be observed through a 6- by 10-inch window above the feeding door. This window was protected by ½-inch mesh hardware cloth that prevented the lamb from smearing it.

A larger door (10 by 14 inches) on the right side of the front surface allowed easy access to the

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* Produced by the Fibra-glass Boat Co., Waverly, Neb.

† Filterdown Pad No. 50 F.G., American Air Filter Co., Louisville, Ky.

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inside of the unit. A small trough for grain or other dry feed was attached to the inside of the large door with two bolts. A drain was installed on the floor near the front to permit the withdrawal of accumulated fluids. This opening was closed by a clamp on a short piece of rubber tubing attached to the drain pipe.

Anesthesia.—Anesthesia was accomplished by hoisting the ewe by the hindlegs and lowering the head for 60 seconds into a steel drum where approximately 3 lb. of dry ice had been pulverized. The use of CO_2 permitted salvage of the carcass for human food.

Hysterectomy.—The abdomen was scrubbed with warm water and, while the anesthetic was being administered, the abdominal surface was further cleansed with a quaternary ammonium antiseptic solution. Immediately after the animal was removed from the steel drum, an incision was made on the midline of the abdomen extending from the anterior edge of the udder to the xiphoid cartilage of the sternum. Simultaneously, the hysterectomy hood was rolled into place so that when the exteriorized uterus and attachments were severed, the gravid uterus rolled into the antiseptic water lock.

Within the sterile hood, operators with scrubbed hands quickly lifted the uterus into the hood, tore it open, and removed the lamb or lambs from the enveloping membranes. The umbilical cord was clamped off with an electrician's alligator clip, and excess mucus was removed from the lamb's mouth and nostrils with a sterile towel. To insure inflation of the lungs, air was forced into the respiratory tract by a ½-inch rubber tube attached to a Tycos rubber bulb and valve which was inserted into the lamb's mouth. Air was pumped into the lungs while the mouth and nostrils of the lamb were held closed. Expansion of the lungs insured the beginning of the breathing process and aided in clearing the passages of heavy mucus.

The umbilical cord was ligated with a short piece of string and cut about 1 inch from the abdomen. The end of the cord was painted with a saturated solution of alum in tincture of iodine. The lamb was dried with sterile towels. Identification, birth weight, and other measurements were recorded before the animal was transferred from the hood.

Isolation.—Each lamb was transported from the hysterectomy hood to an isolation unit inside of two sterile cotton bags, the outer bag being removed before entering the isolation area. Isolation units described by Young and Underdahl[4] were used temporarily for the first lambs. Later, the larger fiberglass units were constructed as described previously (under "equipment").

Nutrition.—The lambs were first fed, about six hours after birth, a liquid diet consisting of a raw egg and 5 ml. of a mineral mixture[5] added to a quart of pasteurized, homogenized, cows' milk. This milk was repasteurized for the first few days. The lambs were fed 2 to 3 oz. of the warmed milk diet in a shallow pan three times during the working day. The lambs needed assistance to find the milk during the first day or two. The diet was later increased to include sterilized tepid water and whole dry oats.

RESULTS

Of the 11 ewes bred for this study, 2 lambed naturally prior to the scheduled hysterectomy. The remaining 9 ewes were

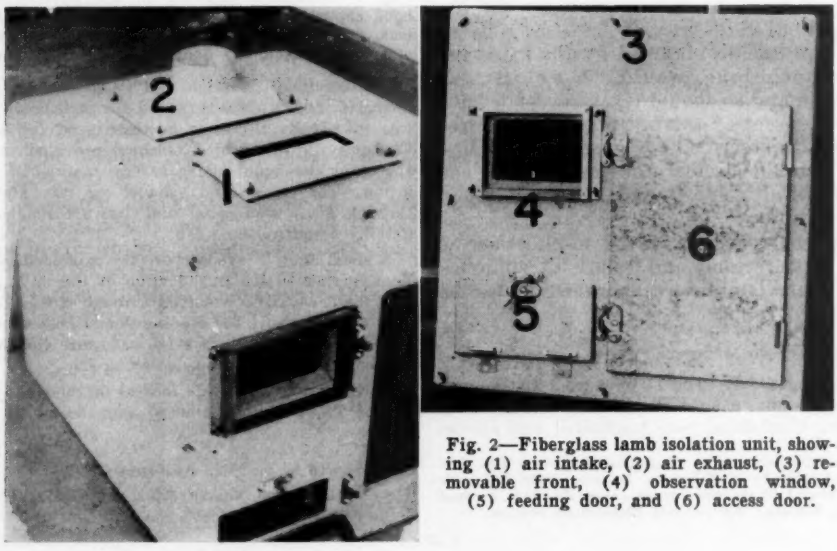

Fig. 2—Fiberglass lamb isolation unit, showing (1) air intake, (2) air exhaust, (3) removable front, (4) observation window, (5) feeding door, and (6) access door.

delivered of 11 lambs by hysterectomy. Although manual artificial respiration was given, 4 of the lambs died immediately, due to respiratory failure. After the adoption of the technique of inflating the lungs with forced air, there were no more losses.

After hysterectomy, the 7 living lambs were transferred to the isolation units and there made satisfactory growth. No digestive or other metabolic disturbances were experienced by the lambs.

DISCUSSION

Since sheep are susceptible to many of the common diseases of cattle, they can be used for the investigation of the causative agents. Establishment of virus diarrhea,[3] mucosal disease,[2] and other viral agents in sheep indicates that they would be satisfactory for preliminary studies of these diseases. Lambs obtained by hysterectomy and kept in isolation are protected from exposure to environmental infection and therefore provide research workers with uniformly susceptible animals needed for experimental studies.

Lambs can be procured by this technique at comparatively low cost, and their size permits easy handling and confinement of large numbers in individual isolation in a small area. Although the breeding season of sheep is limited, lambs can be procured during seven to eight months of the year by proper management and breed selection.

Variation in the length of the gestation period (147 to 152 days) made it necessary to schedule hysterectomy well in advance of calculated parturition. Two ewes lambed naturally 145 and 146 days after breeding, several hours before the scheduled hysterectomy. Lambs delivered by hysterectomy 142 and 144 days after conception were raised successfully. It suggested that the 144th day of gestation could be selected as the time to schedule hysterectomy.

SUMMARY

A method of protecting lambs from environmental infections by use of a hysterectomy technique is described. Of 11 lambs from 9 ewes, 7 were raised successfully in sanitary isolation. Lambs delivered by hysterectomy six and eight days prior to term and raised in isolation provide the research worker in large animal diseases with a uniformly susceptible experimental animal.

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The Effect of Intravenous Glucose Injections on Appetite in Adult Ewes

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STIMULATION and ablation experiments have firmly established the presence, in mice, rats, cats, monkeys, sheep, and goats, of centers in the ventral hypothalamus which function in the regulation of feeding behavior.[1, 2, 4, 6, 8] Little is known, however, concerning the afferent and efferent connections in this brain region; the nature of stimuli which initiate the seeking of or indifference to food; or the location of primary sensory receptors which activate the center. It is not clear whether the hypothalamic neurons function as primary sensory receptors, or merely serve in an integrative capacity to regulate feeding.[6] The presence, in the hypothalamus, of neural elements performing both functions seems likely.

The glucostatic theory of the day-to-day or short-term regulation of food intake proposed by Mayer[10] postulates that receptors are located within the hypothalamic satiation center itself. Injections of gold thioglucose into mice caused severe and selective lesions in the region of the ventromedial hypothalamic nucleus, accompanied by hyperphagia and obesity. The area damaged was that previously postulated by Mayer to contain glucoreceptors. Gold thiomalate and other gold compounds at i.d.$_{50}$ levels produced no hypothalamic lesions and no hyperphagia or obesity. These findings infer that ventromedial hypothalamic neurons are inordinately sensitive to the glucose moiety. The incorporation by these neurons of the glucose with an attached gold atom results in their selective degeneration.

From experiments on man, Stunkard *et al.*[21] reported that intravenous glucagon (the hyperglycemic-glycogenolytic pancreatic factor) abolishes hunger contractions and subjective hunger. Blood sugar was simultaneously raised and capillary-venous glucose difference increased. Both hunger contractions and subjective hunger returned with the subsequent fall in blood glucose levels and decreased capillary-venous difference.

The following relationships are presented by Mayer[10] as important reasons for proposing glucose as the chemostatic regulator of feeding behavior: (1) Carbohydrate metabolism has a central role in the regulation of energy metabolism. (2) Glycogen stores are small and the first to become depleted under starvation conditions. (3) Glucose is used preferentially by all tissues and exclusively by the nervous system. (4) Carbohydrate metabolism is the central regulator of both protein and fat metabolism.

That the ruminant exhibits significant deviations from mammals with simple stomachs, with respect to carbohydrate metabolism, is abundantly clear. Reid[16] reported blood-glucose values in sheep from 20 to 50 mg./100 ml. and little fluctuation in glucose levels associated with feeding. Arteriovenous differences (carotid-jugular) were always less than 2 mg./100 ml., indicating that little glucose was utilized by the peripheral tissues.

In a recent study, Annison *et al.*[4] stated that "blood glucose concentrations in portal and arterial blood of sheep showed little variation throughout the sampling periods, the portal-arterial differences being negligible."

The sheep is resistant to insulin injections, clinical hypoglycemic signs only being manifest when blood-glucose levels of less than 2 mg./100 ml. are reached.[18] It seemed important, therefore, to determine whether variation in blood-glucose level is associated with variation in appetite, as indicated by the feeding behavior of ruminants. Accordingly, a number of adult ewes were given glucose intravenously in order to elevate the blood-sugar concentration for a period during which data regarding food intake and feeding behavior could be obtained.

A depression of rumen motility following intravenous glucose in sheep has recently been described.[23] Since this inhibition was most marked from one to two hours after injection, it was decided to offer food to the animals during this period while maintaining a high blood-glucose level by an additional injection just prior to feeding. This procedure was adopted on the basis of an analogy between ruminal motility and gastric hunger contractions in man.

MATERIALS AND METHODS

In this study, 8 unmated yearling Suffolk ewes, weighing 128 to 132 lb., were used. They were confined in a pen for a pre-experimental period of one

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week, and accustomed to a regimen of approximately 5 lb. of pelleted feed at 11 a.m. daily. These pellets, used successfully for feeding cattle for five years,[12] were readily eaten by the sheep.

Four appetite experiments were conducted in 20 days. Two of the 8 ewes used as simple controls were picked randomly, assigned separate pens, and offered feed at the same time and in the same amounts as were the remaining 6 ewes. Blood samples were not taken from the ewes used as simple controls.

Of the remaining 6 animals, 2 were randomly chosen as controls without intravenous infusion; however, blood samples were collected, feed offered, and data collected on feed consumed. The other 4 ewes were given intravenous infusions of 50.0 per cent glucose or 0.9 per cent NaCl. On occasion, however, it was necessary to deviate from this procedure, due to trauma at the time of infusion. This resulted in somewhat unequal numbers in the treatments. Also, two days' observations were included where all animals were used as simple controls, to provide a more reliable base level of feed intake.

Blood samples were taken prior to and 30, 60, 90, 120, and 360 minutes after the intravenous infusion of 50 per cent glucose or 0.9 per cent NaCl. Immediately after the 60 minutes blood sampling, a further infusion was given. Then all 8 ewes were offered the pelleted ration, and the amount of feed consumed during the next hour was recorded. Urine specimens were obtained periodically.

As the experiment was repeated every four or five days, some ewes were given a variety of treatments. Of four saline solution treatments, two consisted of 300 cc., one of 500 cc. and one of 1,000 cc. Seven 50 percent glucose treatments ranged from 200 cc. to 1,000 cc. All infusions were given in two equally divided doses, at 0 and 60 minutes, respectively. Observations were also recorded from seven "sampled" controls and 18 "feeding" controls.

The oxalated blood samples were always deproteinized on the day of collection (usually within a few hours) using the Somogyi zinc hydroxide method[20] in order to eliminate non-fermentable reducing substances. Glucose concentrations in the filtrates were then determined according to Sunderman's method.[22] Semiquantitative tests for sugar were made on the urine specimens collected, using Benedict's solution and employing the procedure recommended by Osgood.[13]

RESULTS

Blood samples from the control animals yielded average blood-glucose levels of 43.7 mg./100 ml. at the first sampling, and 49.6 mg./100 ml. at the end of the trial two hours later. The animals which were given saline solution injections had mean blood-glucose levels of 51.7 mg./100 ml., which were not significantly different from the controls. However, the glucose-injected animals showed marked increases in their blood glucose levels from their initial levels of 40 mg./100 ml. (table 1). The increase in blood glucose concentration varied with the amount of glucose injected.

Since the second injection of glucose was administered immediately following the 60 minute blood sample, the 120 minute levels for blood glucose in the glucose-treated group may be considered the minimum values during the feeding period (table 2).

TABLE 1—Blood-Glucose Levels of Ewes With and Without Infusions of 0.9 Per Cent NaCl Solution or 50 Per Cent Glucose

Group	Blood sugar (mg./100 ml.)				
	0*	30*	60*	90*	120*
Control	42	....	....	....	53
	30	....	....	....	37
	35	....	....	....	36
	39	....	....	....	53
	45	....	....	....	44
	58	....	....	....	67
	57	....	....	....	65
Saline solution injected (0.9%)					
2 × 150 ml.	44	45	51	55	56
2 × 150 ml.	45	45	43	52	47
2 × 250 ml.	34	50	53	51	60
2 × 500 ml.	49	52	52	....	65
Glucose injected (50%)					
2 × 100 ml.	50	180	150	255	175
2 × 100 ml.	39	315	255	345	235
2 × 150 ml.	44	255	115	440	285
2 × 150 ml.	33	250	180	410	290
2 × 150 ml.	34	360	290	380	290
2 × 250 ml.	....	350	225	510	410
2 × 500 ml.	40	880	600	.....	1,480

* Minutes after treatment.

Feed consumption values were compared directly and the amounts of glucose given per sheep calculated from the total volume of 50 per cent glucose administered, the body weights of all ewes being in the range of 128 to 132 lb. (58.2–60.0 kg.).

The mean feed consumption for the simple controls was 1.5 lb., which was 0.3 lb. less than for the sampled controls. Mean feed consumption values for ewes given glucose were, with one exception, uniformly increased over the mean for sampled control or saline solution-injected ewes, but the difference was not statistically significant (table 2). One ewe, given the highest dosage of glucose (8.3 Gm./kg. of body weight) with a minimum blood-glucose level of 1,480 mg./100 ml. during the feeding period, consumed 1.2 lb. of feed as eagerly as any of the other animals. This dose level exceeds the 1.0 Gm./kg. reported by Vallenas[24] to depress rumen motility.

Urine specimens collected from the control and saline solution-injected ewes were uniformly negative for reducing substances

with Benedict's reagent, while those from the glucose-injected sheep during the feeding period gave strong reactions.

Every attempt was made to prevent disturbance of the animals during the feeding period. Their feeding behavior was observed as carefully as was practicable under these circumstances, and in no case could any difference be detected in the motor responses exhibited when feed was offered nor at any time during the subsequent hour. All animals, regardless of treatment, consumed an estimated three quarters of the total within the first 15 minutes after the pellets were offered.

TABLE 2—Feed Consumption and Minimum Blood Glucose Levels During the Feeding Period

Group	No. of animals	Feed consumed (lb.)		Minimum blood glucose level	
		Average	Range	Average	Range
Simple control	21	1.5	0.5–2.0	.....	.....
Sampled control	7	1.8	0.5–2.6	51	36–67
Saline injected	4	1.9	1.3–2.4	57	46–65
Glucose injected					
2 × 100 ml. (1.67 Gm./kg.)	2	1.9	1.8–2.0	205	175–235
2 × 150 ml. (2.5 Gm./kg.)	3	2.4	2.3–2.5	288	285–290
2 × 250 ml. (4.17 Gm./kg.)	1	2.4	.....	410	.....
2 × 500 ml. (8.3 Gm./kg.)	1	1.2	.....	1,480	.....

Thereafter, eating was intermittent and of a nibbling character. In no case was any hesitation or indifference to the feed observed in any of the ewes in this study.

DISCUSSION

The experimental findings indicated that increased blood-glucose levels in sheep did not decrease feed consumption during the hour following the second glucose administration. This substantiated the inferences to be drawn from the data presented by Reid, and other workers, and on this basis glucose can not be regarded as the key metabolite in short-term appetite control of ruminants.

The glucostatic theory of hunger state does not appear to fit the requirements for regulating a ruminant's day-to-day energy balance. As an alternative, hunger or the hunger state may be considered as associated with respiratory quotients of the order of 0.7, and hence with the burning of fat.[10] On this basis, compounds other than glucose should be considered as possible regulators of appetite in ruminants.

Acetic acid constitutes a possible metabolic alternative to glucose in the regulation of feed intake in ruminants. Acetic acid plays an especially prominent role in ruminant digestion and metabolism: (1) It is well established as an important product of the microbial degradation of cellulose in the rumen;[15] (2) it is absorbed as the predominant source of energy from the rumen, averaging more than 80 per cent of the total volatile fatty acids in the portal vein on a molecular basis;[4] (3) it shows appreciable arteriovenous differences;[17] and (4) it is stored in slight amounts (this may require elaboration in view of the availability of acetyl CoA from long-chain fatty acids. The important point, however, is that this is not a preferred pathway, but proceeds under conditions of starvation with a respiratory quotient of 0.7, and also requires the continuous utilization of amino acids from tissue protein in order to supply the tricarboxylite acid cycle intermediates. Thus, fat does not represent a reserve supply for acetate in the same sense that glycogen does for glucose.)

As a metabolic regulatory agent, therefore, acetic acid appears to have many desirable characteristics. However, it can not supply a source for the glucose needed as muscle and liver glycogen, and perhaps for glucose utilization by the central nervous system, nor does it form the necessary intermediates for the operation of the tricarboxylic acid cycle. Therefore, acetate is a controlling factor in neither the regulation of protein metabolism nor even its own oxidation. These are serious deficiencies and, unless they can be resolved, acetic acid must be eliminated as a satiation stimulant.

As previously mentioned, by far the greater proportion of glucose formed in ruminants is via gluconeogenesis, and its precursor under nonstarvation conditions is generally agreed to be propionic acid [11,14] which is absorbed in significant quantities from the rumen. Consequently, this compound performs precisely those functions in which acetic acid was found to be deficient. Propionate, however, also appears deficient as a regulatory metabolite for the following reasons: (1) The highest mean propionate value reported by different authors [3,4,11,17] was less than 0.6 mg./100 ml. in the peripheral blood, and a more recent report, using vapor-phase chromatography to separate the volatile acids of sheep blood

demonstrated only occasional traces of this three-carbon homologue peripherally. Since nearly all values in peripheral blood were zero, regardless of ration, while portal concentrations ranged from 6 to 11 per cent on a molecular basis, it is apparent that propionate is selectively removed as the blood passes from the hepatic portal system and through the liver to the systemic circulation. Only under extreme conditions, such as the infusion of large amounts of pure propionic acid into the rumen, does the portal concentration of this absorption product exceed the capacity of the liver to extract it. Hence the central nervous system is seldom subjected to variation in concentration of propionic acid. (2) The quantity of propionic acid absorbed from the rumen and metabolized in the liver provides less than one tenth of the number of calories required to maintain heat production in sheep (computed from data of Armstrong and Blaxter,[5] Annison *et al.*,[4] and Schambye [19]).

A chemostatic hypothesis for regulation of energy exchange in ruminants, if a mechanism similar to that of glucostatic hypothesis is implied, leads to difficulties due to the lack of a metabolite possessing characteristics which seem necessary. Acetic acid represents the predominant energy source and is strategically located in the metabolic pathway, although the essential tricarboxylic acid cycle intermediates and glucose are not obtainable from acetate. Propionic acid, however, provides these essential intermediates, but appears to be absorbed in quantities so small that it can not constitute an important source of energy. Propionate, in addition, can hardly show arteriovenous differences peripherally, since the compound is essentially absent in the peripheral vascular system. Other metabolites are equally deficient in properties deemed requisite as a regulator of appetite.

Since no single metabolite appears to be the controlling factor, it is conceivable that some combination of metabolites, or the integrated effects of several metabolites, may exert the control. To establish the essential metabolites, if any, for control of feed intake will require extensive studies of the intake before, during, and after the intraruminal or intravenous administration of carbohydrate metabolites, amino acids, and possibly even compounds more distantly related to the citric acid cycle.

SUMMARY

The effects of intravenous infusion of glucose on feed intakes were investigated in adult ewes. Infusions of from 1.67 to 8.3 Gm. per kilogram of body weight were given over a two-hour period and did not affect the food consumption of the ewes or their feeding behavior. These observations cast some doubt upon the possible role of glucose as the regulating mechanism in short-term appetite control in ruminants. Possible alternative mechanisms were discussed.

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Copper and Iron in Livers of Undernourished Cattle and Sheep from Northeastern and Northern Brazil

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THE LIVER is the main storage organ for iron and copper and the determination of these elements gives an index of the iron and copper status of the animal body. A range of clinical conditions which develops in different species is a result of a dietary deficiency of these trace elements.

Low copper values in livers of cattle and sheep are known to occur only due to the following dietary factors: (1) the amounts of copper in the diet and (2) excessive amounts of molybdenum, the latter acting by its limiting effect on liver copper storage. There are still other unknown factors affecting copper retention.[9] High iron values were found in the livers of cattle with copper deficiencies[1] and in sheep with copper and cobalt deficiencies. Feeding iron to sheep resulted in the storage of a large amount of this element in the liver.[9]

The determination of copper and iron in the liver is of great significance to the study of mineral deficiency diseases in animals. Since the cattle of some regions of northern and northeastern Brazil are frequently in a poor nutritional state, it seemed important to determine the iron and copper contents of livers of the animals from that region. In the present work, the results of our analysis regarding copper and iron up to the present time are given in order to establish the possible relationship between the lack of these elements and the general status of the animal.

EXPERIMENTAL PROCEDURE

All liver samples were collected postmortem, with the necessary precautions to avoid chemical contaminations. Stainless steel scissors were used to cut the organ, and 10 per cent formalin solution was used in the preservation of the liver samples. The formalin was formaldehyde reagent A.C.S., code 1778 and the water used for washing the glasses and in the preparation of the 10 per cent formalin solution was twice distilled in glass apparatus.

Livers were analyzed for copper according to the method of Eden and Green[6] by digesting 0.05 to 0.1 Gm. of dry sample with H_2SO_4, HNO_3, and $HClO_4$. The residue of the combustion was diluted to 25.0 ml. and the copper color was developed by a 0.5 per cent solution of sodium diethyldithiocarbamate. The color was then extracted with amyl alcohol and compared in a Unican spectrophotometer at 460 mμ with a standard curve.

TABLE 1—Copper and Iron Values in Livers of Cattle and Sheep of Northeastern and Northern Brazil

Sample No.	Species	Age	Copper*	Iron*
		COAST		
103	Bovine	3 years	9.0	567
104	Bovine	3 years	2.5	993
117	Bovine	3 years	26.0	1340
109	Bovine	3 years	5.0	377
486	Bovine	5 years	0.0	34,858
22	Bovine	7 years	5.0	13,939
4N	Bovine	8 years	35.0	466
266	Ovine	1 month	11.0	719
267	Ovine	2 months	23.0	3,692
471	Ovine	8 months	25.0	3,024
		INLAND		
140	Bovine	1 year	148	549
136	Bovine	2 years	200	1,150
96	Bovine	2 years	150	1,604
533	Bovine	2 years	52	438
524	Bovine	3 years	114	496
46	Bovine	4 years	168	433
324	Bovine	4 years	119	475
78	Bovine	5 years	240	306
171	Bovine	6 years	264	552
192	Bovine	6 years	115	901
154	Bovine	6 years	106	1,400
532	Bovine	7 years	39	347
323	Bovine	9 years	66	547
176	Bovine	10 years	154	1,741
559	Bovine	10 years	75	998
325	Bovine	10 years	80	359
168	Ovine	adult	250	371
182	Ovine	adult	268	1,562

* Values in p.p.m. measured on the dry weight basis.

Iron in livers was determined in the clear digested sample by a modification of the method of Wong[11] which consists of a reaction with potassium thiocyanate and the color extracted with isoamyl alcohol and compared at 510 mμ with a standard curve.

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RESULTS

Data from 10 adult cattle, well developed and in good nutritional state, obtained from Central Brazil (Curvelo area in Minas Gerais), represented the normal values for copper and iron in livers. The mean value for copper was 134 ± 28 parts per million (p.p.m.) and for iron, 454 ± 78 p.p.m. on the dry samples (range for copper 95 to 194 p.p.m. and for iron 381 to 563 p.p.m.). These values are in agreement with those considered as normal by various authors.[2, 3, 6]

The copper and iron values found in livers of cattle and sheep from northeastern and northern Brazil are shown (table 1). Low values of copper were found in all liver samples from cattle and sheep of the coastal area and also in two samples (532, 533) collected from cattle in the plain, far from the coast. Three samples (323, 559, 325) gave values which were on the lower borderline of the range of normal values. The copper values found in the rest of the liver samples were considered normal.

A great number of liver samples (103, 104, 117, 486, 22, 266, 267, and 471) showed high iron values, especially those with low copper values from animals of the coastal area. High iron values were also found in livers with normal or high copper values (samples 136, 96, 192, 154, 176, 559, and 182).

DISCUSSION

Low copper values in the livers of cattle and sheep are known to occur due to dietary factors. The principal dietary factors influencing copper storage, and hence liver copper concentrations, are the levels and proportions of copper, molybdenum, and inorganic sulfate in the diet. There is also evidence of the existence of other unknown dietary factors affecting copper retention. Dick [4] found that, in sheep, manganese is one of these factors. For practical reasons it may be said that it does not matter what the complicated mechanism of copper assimilation and its storage in the liver may be, once the feeding of a copper supplement corrects the copper deficiency of the animal.

The low values of copper in liver samples collected in the coastal areas of northern and northeastern Brazil suggested that, in these areas, cattle are cobalt deficient as in Western Australia,[10] where the "wasting disease" of cattle appears, or in South Australia, where the "coast disease" of sheep is found.[7, 8] The study of cobalt contents in livers of cattle and sheep of these coastal areas will be the subject of a forthcoming publication.

High liver iron values were found by Bennetts *et al.*[1] in cattle with copper and cobalt deficiencies. The feeding of iron to sheep resulted in a large storage of this element in the liver.[9]

The results of the analysis of iron in the liver showed no relationship between the values of copper and iron. High values of iron were found in livers with low and high copper contents, and normal iron values in livers with low copper contents.

SUMMARY

Analysis of the copper and iron values in livers of cattle and sheep from northeastern and northern Brazil are given.

The copper content of livers was consistently low in all samples collected from cattle as well as from sheep in the coastal area of northern Brazil, where mineral requirements were inadequate.

Many liver samples showed high iron values which could only in part be related to the simultaneous occurrence of low copper values.

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A Partial Chemical Analysis of the Mucoprotein of Siliceous Urinary Calculi of Bovine Origin

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AN EARLY REPORT from this laboratory [14] described a partial inorganic qualitative analysis of siliceous bovine urinary calculi. More recently, a detailed quantitative analysis of the inorganic constituents has been made by Dang.[5] He found that the average silicon concentration (as SiO_2) of a large number of typical siliceous stones of bovine origin was in the range of 50 to 60 per cent on an air dry basis. He further reported that the organic matrix predominantly represented protein, as calculated from total and amino nitrogen values. The matrix was thought to be mucoprotein, as evidenced by mucicarmine staining, and was found to lie generally in discrete laminations throughout the stones.[15] The possibility that the mucoprotein matrix is of considerable importance in the formation of the stone has been suggested by Boyce and Garvey [2] and Boyce *et al.*[3] An analysis of the amino acid and carbohydrate composition of the mucoprotein matrix of calculi from human beings has been reported by King and Boyce.[9]

This paper reports the results of experiments in which the chemical composition of the mucoprotein(s) of siliceous bovine calculi has been partially determined.

MATERIALS AND METHODS

Bovine calculi of the mulberry type, collected from range cattle in Montana, were used throughout. Calculi of this type are predominantly silica and mucoprotein with small amounts of other inorganic materials. All calculi were air dried and powdered before use. Dialyzed urine sediment was prepared by exhaustive dialysis of bovine urine (pooled three-hour collections from 4 steers) and subsequent evaporation.

The lack of suitable chemical techniques for the separation of the organic matrix from the inorganic phase of siliceous calculi made it necessary to perform the separation, where required, after hydrolysis.

Tests for Sugars, Amino Sugars, and Other Carbohydrates.—Samples of air-dried, powdered calculus material from pools of ten to 15 individual stones were hydrolyzed in N H_2SO_4 for four hours at 120 C. For sugar chromatography the hydrolysates were desalted by a number of techniques. The pyridine extraction technique of Malpress [11] was used. Ion-exchange treatment with resins IR 120 H^+ and IRA 410 acetate$^-$ was also tried.

The microdesalter method of Zweig and Hood [18] was used for samples spotted on paper. For the latter it was necessary to add the appropriate amount of $BaCl_2$ to the hydrolysates and centrifuge out the $BaSO_4$ and inorganic residue from the stone material. The solution containing the sugars and HCl was then repeatedly blown to dryness to remove the HCl. Subsequent desalting on the microdesalter yielded spots fairly free of salt. The chromatography was single or double ascension for 35 cm. each time. Solvents used were butanol:acetic acid:water (4:1:5), and *n*-propanol:ethyl acetate:water (7:1:2). The chromatograms were sprayed and dipped in benzidine-acetate [1] or aniline-phthalate.[1]

The method of hydrolysis used for sugars was found satisfactory for maximum hexosamine yield also. Tests for hexosamine were run on such hydrolysates by the Elson-Morgan [17] and the indole HCl [6] methods. The methods of Winzler were used to determine sialic acid,[17] and to measure hexose by the orcinol test.[17] Both the Molish [6] and the general cysteine H_2SO_4 reactions [6] were used to test for sugar class. The carbazole method [6] was used to test for hexuronic acids.

Test for Amino Acids.—Samples of individual stones and a pool of four stones were hydrolyzed in 6N HCl for seven hours at 120 C. Desalting was accomplished by acetone/HCl extraction [7] of the hydrolysate after it had been blown to dryness by an air jet. Two-dimensional paper chromatography was used with a double ascension each way in either of the following two pairs of solvent systems: sec-butanol:3 per cent NH_4OH (5:2) and sec-butanol:formic acid:water (15:3:2), or *n*-butanol-acetic acid:water (4:1:5) and *n*-butanol:ethanol:water (4:1:1). Color development was achieved by dipping in a solution of ninhydrin in acetone [16] followed by a 60-minute development at room temperature and then a 30-second dip in 100 C. paraffin. Densitometer scanning was used to give a quantitative measure of each amino acid.

All colorimetric analyses and spectral curves were obtained using a Coleman senior spectrophotometer.

From the Montana Veterinary Research Laboratory (Montana Experiment Station and Livestock Sanitary Board cooperating), Bozeman. Paper No. 443, journal series, Montana State College, Agricultural Experiment Station.

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RESULTS

Spectral curves run on four pooled stone samples and the dialyzed urine sediment using the general cysteine H_2SO_4 reaction at five minutes, 30 minutes, and 24 hours gave the following results. No deoxypentose appeared to be present in any of the pools tested, with the possible exception of the dialyzed urine sediment, as there was no evidence of a 375 mμ peak after 24 hours. No heptose appeared to be present as there was no rapidly forming 430 mμ peak. A stable peak was seen in the 410 to 415 mμ range, indicating the presence of hexoses. Since the 390 mμ minus 424 mμ value decreased in 24 hours, it was likely that pentose was present. However, since this value did not decrease to a small fraction of its original, it was concluded that methyl pentose was also present.

The results of the Molish test run on the same samples indicated the presence of methylpentose and hexose due to the peak between 560 and 570 mμ. A slight shoulder at 550 mμ was indicative of some pentose in the stone pools. A marked peak was evident in the dialyzed urine sediment at 550 mμ, indicating a high concentration of pentose.

The test for hexuronic acid was inconclusive, due to the high background in the violet end of the spectrum, and perhaps would be unreliable in any event.[11]

Spectral curves for hexose (graph 1) using the orcinol test were run and compared with a mannose/galactose standard. The standard and unknowns matched well, and the quantitative results for the percentage of hexose in stones are shown (table 1).

TABLE 1—Hexose in Pooled Samples of Siliceous Urinary Stone Material and Dialyzed Urine Sediment

Material analyzed	Hexose (air-dry basis)
Group A (19 stones)	0.56%*
Group B (10 stones)	0.81%
Group C (12 stones)	0.87%
Dialyzed urine sediment (pooled three-hour collections from 4 steers)	1.39%

* Each value represents a single analysis of the pooled material.

The spectral curve of the diphenylamine test for sialic acid (graph 2) revealed the 525 mμ and 700 to 710 mμ maximums and the 570 mμ minimum characteristic of sialic acid for three-stone pools tested and also for the dialyzed urine sediment. The lack of a blue color or of significant absorption in the 600 mμ region again suggested the absence of deoxyribose in these pools. The average percentage of sialic acid in air-dry stone material was estimated to be about 0.03 per cent.

The following results were found in tests for hexosamine. Using the Elson-Morgan method, an average of 0.22 per cent was found in air-dried, pooled stone material with little deviation between three individual pools. Spectral curves showed peaks at 540 mμ both for unknowns and known hexosamine samples. When the length of hydrolysis time was varied from one fourth to four hours, no change in spectral peak was observed, suggesting no three or four substitution in the partially unhydrolyzed polysaccharide.[4] The hydrolysis time for maximum hexosamine yield was from three to four hours under these conditions. The hexosamine value for dialyzed urine sediment (0.29%) was slightly higher than for stone material.

To test the possibility that the color given by the Ehrlich reagent was due to the sialic acid and not to hexosamine, the direct Ehrlich reaction was used. Sialic acid will produce a derivative with Ehrlich reagent directly[17] which yields a color similar to the color produced by hexosamine after pretreatment with acetylacetone and alkali. Using this direct method, however, little color development occurred (less than 5% of the values obtained when pretreatment was included), indicating that the small amount of sialic acid present did not account for the color.

The indole HCl method for hexosamine gave uniform results with six pools of stone material. The average value by this method was 0.19 per cent. It was found necessary, however, to increase the length of boiling time for color development to 15 minutes to obtain maximum values by this method.

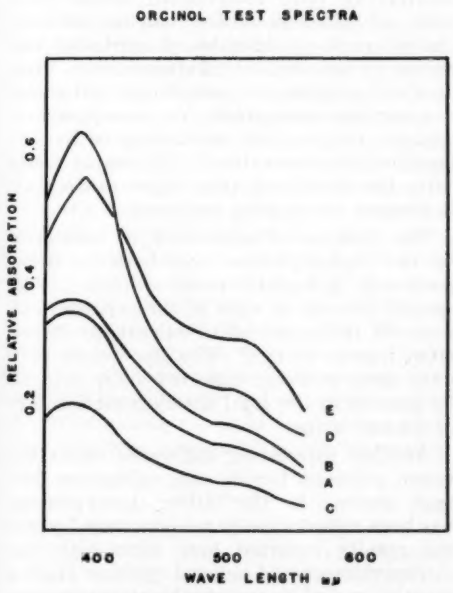
Interesting results were obtained with the Elson-Morgan method when acetonylacetone was substituted for the acetylacetone. Typical color development was obtained with standard hexosamine solutions with a spectral peak at 540 mμ, but with slightly less color per unit of hexosamine. However, with hydrolysates of stone material, values were obtained as high as 25-

fold greater than those when acetylacetone was used. Unlike the standard, the unknowns had a peak between 520 and 530 mμ. Further experimentation revealed the specificity of this modification to be broad. Various proteins, amino acids, and amines gave colors by this method. A detailed description of the specificity and usefulness of this reaction will be described elsewhere.[8]

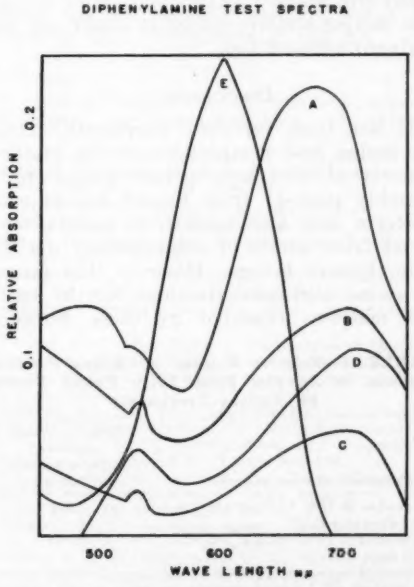
Chromatography for hexoses has tentatively identified the presence of the following in four pools tested in decreasing order of spot intensity: hexosamine (strong), glucose, galactose, and mannose (medium), and rhamnose (weak). The lack of a method for obtaining completely salt-free samples has made sugar chromatography difficult and the results less reliable than would be desired.

The relative amino acid color intensities of four single stones, one pool of four stones, and a bovine urine precipitate (material not precipitable by perchloric acid, but precipitated by phosphotungstic acid) are compared (table 2). While the qualitative amino acid composition of these stones and a large number of other stones tested was the same, there existed marked quantitative differences, as measured by relative color intensities. Furthermore, there existed both qualitative and quantitative differences between the stones and the phosphotungstic acid precipitate which supposedly represents mucoprotein of the urine.

It seemed likely, as a result of some work recently reported by Muir,[13] that one might be able to liberate some of the hexose-positive material from ground stone samples in a form similar to the native state. Various methods were tried. The $CaCl_2$ extraction at pH 6.8 resulted in a greater release of hexose-positive material than any of the other treatments (table 3). Neither the papain nor trypsin treatments resulted in an appreciable release. Trypsin was the best, though only about one seventh as good as the $CaCl_2$ at pH 6.8. The enzyme



Graph 1—Orcinol test spectra given by the following: (A) 0.0493 Gm. of hydrolyzed, pooled stone material from group A; (B) 0.0460 Gm. of hydrolyzed, pooled stone material from group B; (C) 0.0183 Gm. of hydrolyzed, pooled stone material from group C; (D) 0.0334 Gm. of hydrolyzed, dialyzed urine sediment; and (E) 0.67 mg. of a galactose/mannose standard (0.335 mg. of each).



Graph 2—Diphenylamine test spectra given by the following: (A) 0.0500 Gm. of hydrolyzed, dialyzed urine sediment; (B) 0.0493 Gm. of hydrolyzed, pooled stone material from group A; (C) 0.0183 Gm. of hydrolyzed, pooled stone material from group C; (D) 10 μg. of crystalline sialic acid; and (E) 40 μg. of 2-deoxyribose-5-phosphate. (Curves D and E were taken from a reference,[9] reduced 1/10 and corrected for 2-cm. Coleman cell.)

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TABLE 2—A Comparison of the Relative Intensities* of the Ninhydrin-Developed Amino Acid Colors of Hydrolysates of Single Stones, a Stone Pool (Four Stones), and a Phosphotungstic Acid Precipitate (PTA) of Bovine Urine

Amino acid	Stone A	Stone B	Stone C	Stone D	Pool	PTA precipitate
Leucine	3.2	5.8	2.6	3.2	4.3	2.7
Isoleucine	1.1	5.8	1.4	3.7	1.7	1.2
Phenylalanine	2.6	T†	T	T	T	T
Methionine	T	T	T	T	T	No
Valine	4.3	5.8	3.4	5.5	5.6	5.2
Tryptophane	T	T	T	T	T	No
Tyrosine	1.0	3.2	T	4.4	8.9	1.8
Proline	T	T	T	T	T	T
Alanine	17.6	11.5	11.5	19.1	17.1	7.6
Threonine	7.2	13.5	8.9	7.9	8.2	7.2
Glycine	11.5	14.7	11.3	10.2	6.0	13.5
Serine	17.6	26.0	15.4	14.4	9.8	21.7
Glutamic acid	13.0	10.1	15.8	9.9	11.9	15.9
Aspartic acid	13.2	6.0	19.7	9.7	13.8	13.1
Cystine	1.7	1.6	5.0	3.5	4.8	2.0
Lysine/arginine	6.0	1.6	4.9	8.3	7.2	7.5

* The intensities are expressed in arbitrary units of relative color intensity of each amino acid as determined by densitometer scanning, and are approximately equal to the percentage of each amino acid present in the hydrolysate. † T indicates a trace (below 1%) except for proline, whose yellow color was not quantitatively assayed.

treatments were used on the assumption that the hydrolysis of the protein might result in release of bound polysaccharide material. Only the trypsin treatment, however, showed much hydrolysis as judged by ninhydrin assay [12] of released amino acids. The hexose-positive material could not be dialyzed without loss.

DISCUSSION

It has been reported previously [9] that the amino acid composition of the matrix material of calcareous urinary stones (presumably pooled) from human beings was uniform and also similar to matrix material from stones of submaxillary glands from human beings. However, the range of amino acid concentrations for the various matrices reported by these workers seems wide for the justification of such a conclusion, especially if the material was from pools of a number of stones, as this would tend to hide any differences between individual stones. Our results in comparing both individual stones and pools of siliceous bovine calculi indicate that there is considerable quantitative variation in the amino acid composition. This does not preclude the possibility that there is a certain mucoprotein, or mucoproteins, common to all stones which may be important in stone formation.[2,3] It simply indicates the likelihood that other protein(s) is present in varying amounts.

TABLE 3—Relative Release of Hexose-Positive Material in Samples From Fifty Pooled Stones by Various Treatments

Treatment of stone material	Relative hexose released (optical density at 540 mμ)
Suspended in 10% $CaCl_2$ at pH 6.8 (2 days at 4 C.)*	0.870
Suspended in 10% $CaCl_2$ at pH 9.5 (2 days at 4 C.)	0.355
Suspended in papain, buffer, 0.02 M acetate at pH 5.4, with cysteine and EDTA† (18 hours at 45 C.)	0.105
Suspended in trypsin, buffer, 0.02 M veronal at pH 7.7 (12 hours at 25 C.)	0.120
Suspended in water (2 days at 4 C.)	0.070
No stone material (papain blank)	0.085
No stone material (trypsin blank)	0.050

$CaCl_2$ extraction at pH 6.8 resulted in release of over one half the total hexose of stone material.
† Ethylene diamine tetracetic acid.

The presence of sialic acid, as indicated by the diphenylamine test both in stone pools and in dialyzed urine sediment, is of special interest in view of the reported absence of this material in calcareous stones from human beings.[9] The same authors [10] have more recently reported sialic acid to be present in the total nondialyzable solids of human urine.

Another interesting difference exists between siliceous bovine and calcareous human stones. In the latter, deoxypentose has been reported to be a component,[9] while the results reported here with both the diphenylamine and general cysteine H_2SO_4 reaction reveal no measurable deoxypentose in siliceous bovine stones.

The inability to demonstrate conclusively the presence of hexuronic acid does not necessarily indicate its absence from stone material. Hydrolytic destruction and dialysis loss,[10] as well as other factors, such as

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the high background in the violet end of the spectrum, can cause erroneous results.

Using the total nitrogen (2.5%) and amino nitrogen (0.9%) values reported by Dang [5] and the nitrogen accounted for by the hexosamine and sialic acid reported in this paper, one finds about 1.5 per cent nitrogen (of the total stone weight) unaccounted for. As yet there is no indication of the nature of the compound(s) in which this nitrogen is found. This problem is being investigated further.

An interesting contrast can be drawn between the chondroitin sulfate complex reported by Muir [13] to be extracted from hyaline cartilage by $CaCl_2$, and the hexose-positive material released from stone samples by the same extraction procedure. While the former is nondialyzable, the latter is rapidly lost on dialysis. Thus the molecular weight of the extracted hexose positive material from stones must be less than the chondroitin sulfate complex. The nature of the hexose-positive material released by $CaCl_2$ extraction is now under investigation.

With the partial release of hexose-positive material from ground stones using $CaCl_2$, the possibility of chemically characterizing the organic material of siliceous bovine calculi now seems less remote. If $CaCl_2$ extraction and other suitable methods actually yield a relatively large molecular weight fraction(s) unchanged from the native state, then characterization of such a fraction(s) should allow comparison with urine constituents and ultimately reveal the components of urine involved in the organic matrix of stones. If the organic matrix does play the important role assigned to it by some workers [2,3] in the cause of urinary stones, then such further characterization should lead to the information required in devising suitable measures to prevent stone formation.

SUMMARY

A partial analysis of the chemical composition of the mucoprotein of siliceous urinary calculi of bovine origin has revealed the following:

1) General tests for sugar class (Molish and cysteine-H_2SO_4) indicated the presence in stone material of hexose, pentose, and methyl pentose. Neither deoxypentose nor hexuronic acid was found.

2) Spectral curves for the standard and unknowns using the orcinol test for hexoses matched well. Stone material was found to contain 0.56 to 0.87 per cent hexose (air-dry basis).

3) The diphenylamine test for sialic acid revealed the presence of this material in stone samples at a level of about 0.03 per cent.

4) Tests for hexosamine by the Elson-Morgan and the indole HCl methods showed an average hexosamine content in stones of 0.22 and 0.19 per cent, respectively.

5) Paper chromatography of hexoses in stone material revealed the presence of the following in decreasing order of spot intensity: hexosamine (strong), glucose, galactose, and mannose (medium), and rhamnose (weak).

6) Amino acid chromatography of stone material showed 16 amino acids present in all stones tested. However, there were marked quantitative differences from one stone to another.

7) The hexose-containing material from stones can be partially released by a 10 per cent $CaCl_2$ extraction at pH 6.8.

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The Hematology of Chronic Bovine Reticuloperitonitis

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TRAUMATIC RETICULITIS or reticuloperitonitis remains one of the major diagnostic problems in cattle practice. Many authors have reported on the value of blood examinations as an aid in establishing or confirming the diagnosis.[6, 7, 10, 20] Most reports have been concerned with acute cases. None have reported specifically on chronic cases although they have been included in some reports.

This study was made in an attempt to determine if the hemogram in chronic traumatic reticulitis followed a pattern consistent enough to be of value in diagnosis and prognosis.

Although blood studies may be helpful in diagnosis, the results are not always conclusive and should be considered only as supportive evidence. In field cases, frequently only one blood sample is examined. Each reading is subject to errors in technique, and individual animals may show surprising variations in the blood picture within a matter of hours.

Since the clinical syndrome of chronic cases is less definite than that of acute cases, the detection of the former is often more difficult. Early discovery is difficult except in cows which are milked and observed daily.

Confusing syndromes include: (1) peritonitis from other causes, (2) malignant lymphoma, and (3) displaced abomasum. A representative number of these conditions were included in the study for comparison. Relatively uncomplicated cases of traumatic reticulitis were also compared with those that were complicated by other pathological processes.

MATERIALS AND METHODS

The veterinary clinic at the Iowa State College regularly receives cattle with traumatic reticulitis, in which the condition has been present for one week to several months. To compile this report, ordinary hospital case reports were selected on the basis of: (1) blood count made within two days of admission to the clinic, (2) illness of at least seven days' duration, and (3) diagnosis confirmed by laparotomy or necropsy, or both. Seven days was arbitrarily set as the division between acute and chronic disease. It has been shown that the recovery rate following "hardware operations" is greatly reduced when clinical signs have been present more than seven days.[20]

In most cases, blood samples were taken between 9:00 a.m. and 12:00 noon. In a few cases, blood was drawn immediately after the animal arrived at the clinic; in others, after the animal had rested overnight. These cases were not differentiated. Although sex, breed, time of year, environmental temperatures, state of nutrition, etc., were reported [20] to produce variations in the various blood constituents, these factors were disregarded.

The body temperatures reported were those taken on the morning following admission to the clinic.

Sterile, dry, 10-cc. test tubes, each containing 1 cc. of 2 per cent potassium oxalate evaporated to dryness, were used for collecting blood samples.

Five per cent acetic acid was used as a diluting solution for total leukocyte determinations. The leukocyte counts were made on a double Neubauer "bright line" counting chamber. In filling the counting chamber, a few drops of fluid were discarded from the capillary bore to remove the unmixed fluid. Each of the four corner squares of 1 sq. mm. in area and 1 mm. in depth was counted. Therefore, 4/10 mm. was counted and, since the dilution in the pipette was 1:20, the total leukocytes in the four squares were multiplied by 50. This figure represented the leukocytes per cubic millimeter. Blood smears were prepared with Field's stain modified for blood smears. All neutrophils that showed any evidence of segmentation (having filamentous bands connecting lobes of the nucleus) were interpreted as segmented neutrophils. Cells in which no filaments could be seen were called "stab neutrophils" or "stabs."

Included in the group of 50 cases of relatively uncomplicated chronic traumatic reticulitis were some in which vagal nerve paralysis developed as a secondary complication. Since this appears to result in a mechanical interference with the passage of ingesta rather than an extension of the infectious process, these cases were not excluded from the relatively uncomplicated group.

The complicated chronic traumatic reticulitis group included cases of pericarditis and of ab-

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TABLE 1—Hemograms of 50 Cattle with Relatively Uncomplicated Chronic Traumatic Reticulitis

Case No.	Temp. (F.)	Breed & age	History & duration	Leukocytes (total)	Neutrophils		Lympho-cyte	Eosin-ophil	Mono-cyte	Laparotomy findings
					Stab	Seg.				
1	102.0	Hol. 4 yr.	?*	13,720	4,500† 33.5**	6,100 44.4	2,900 21.1	200 1.4	....	Adhesions of ret. to dia.; no metal penetrating.
2	101.5	Her. 3 yr.	?	10,680	5,000 46.8	1,000 9.3	4,500 42.0	....	....	Adhesions, ret. to dia.
3	102.0	Sh. 3 yr.	?	24,140	5,500 22.7	8,200 33.8	9,600 39.8	800 3.3	....	Wire embedded in wall of ret., surrounded by pus and fibrin, removed.
4	101.2	Hol. 4 yr.	?	6,700	2,000 29.5	1,100 16.4	3,400 50.8	200 3.0	....	Wire penetrating ret. was removed; many adhesions.
5	104.6	Hol. 5 yr.	?	13,480	3,700 27.4	4,200 31.3	5,100 37.8	400 3.0	....	Extensive adhesions in ret.-dia. area; nail penetrating ret. was removed.
6	103.0	Hol. 5 yr.	?	13,820	4,200 30.4	5,900 42.6	3,500 25.4	200 1.4	....	Many adhesions between rumen and ret. and dia.; 4-in. wire removed, both ends had penetrated ret.
7	.....	Hol. 7 yr.	Anorexia, several times in past month.	6,780	200 3.0	2,200 32.4	4,200 62.0	100 1.5	....	Adhesions from ret. to dia. and abdominal floor; two nails embedded in ret. were removed.
8	102.0	Hol. 5 yr.	Anorexia, 1 week.	7,900	2,900 36.6	2,800 35.2	2,200 27.8	....	....	Many adhesions between ret. and abdominal wall; several fistulous tracts involving ret.
9	102.0	Hol. 3 yr.	Calved 3 wk. ago	11,340	1,500 13.0	4,500 40.3	4,700 41.4	100 0.9	500 4.3	Nail penetrating floor of ret. was removed.
10 (v.p.)‡	100.4	Hol. 3 yr.	?	5,360	1,500 28.0	2,100 39.1	1,500 28.0	100 1.9	100 1.9	Reticular wall adhered to dia.; wire perforating ret. and dia.
11 (v.p.)	101.2	Hol. 5 yr.	Calved 4 mos. ago—anorexia, 1 wk.	7,160	3,100 43.3	1,900 26.6	1,400 18.4	800 11.1	....	Adhesions; wire penetrating floor of ret. was removed.
12	100.6	Hol. 4 yr.	Calved 4 wk. ago.	4,880	1,200 25.7	1,100 23.3	1,800 38.5	600 12.8	100 2.1	Adhesions of ret. to dia.; no foreign bodies in ret.
13	104.4	Hol. 6 yr.	?	4,100	2,100 51.2	100 2.4	1,500 36.5	400 9.7	....	Ret. adhered to dia. and abdominal floor; 2 penetrating wires removed from ret.
14	.....	Hol. 2 yr.	?	10,200	3,100 30.4	1,600 15.7	4,800 47.0	500 4.9	200 1.9	Many adhesions near dia.; wire penetrating ret. was removed.
15	104.4	Swiss 5 yr.	?	12,780	3,100 24.0	5,700 44.5	3,800 29.9	200 1.5	....	Adhesions; 3 nails and a wire penetrating ret. were removed.
16	101.0	Hol. 5 yr.	Anorexia, 10 days	16,000	2,200 13.7	4,700 29.0	9,000 56.2	....	100 0.5	Adhesions forming; wire penetrating ret. was removed.
17	101.6	Swiss 1½ yr.	?	25,000	13,800 54.8	6,600 25.2	4,600 17.8	....	500 1.9	Many adhesions involving ret.; wire penetrating ret. was removed.
18	101.8	Hol. 3 yr.	?	14,640	3,100 21.1	3,900 26.6	5,900 40.4	1,900 13.0	100 0.7	Adhesions of ventral surface of ret.; wire in adhered mass could not be removed.
19	103.0	Hol. 3 yr.	?	7,200	1,000 13.9	2,400 33.2	3,300 46.0	300 4.1	200 2.8	Adhesions of ret.; penetrating wire could not be removed.
20	100.6	Hol. 2½ yr.	?	17,740	8,400 48.0	3,300 19.0	5,200 29.0	500 2.4	300 1.6	Ret. adhered to abdominal wall; wire penetrating ret. removed.

Seg. = segmented; ret. = reticulum; dia. = diaphragm; Hol. = Holstein-Friesian; Her. = Hereford; Sh. = Shorthorn; Guern. = Guernsey.

* Question mark indicates that the condition was present for a minimum of one week.

† Expressed as No. per cubic millimeter. ** Expressed as percentage of total leukocytes.

‡ Concurrent vagal paralysis, proven at necropsy.

(Table 1 continued on next page)

TABLE 1 (Continued)—Hemograms of 50 Cattle with Relatively Uncomplicated Chronic Traumatic Reticulitis

Case No.	Temp. (F.)	Breed & age	History & duration	Leukocytes (total)	Neutrophils		Lympho-cyte	Eosin-ophil	Mono-cyte	Laparotomy findings
					Stab	Seg.				
21	100.6	Hol. 4 yr.	?	8,040	1,200 14.9	1,200 14.9	5,300 66.0	200 2.5	100 1.2	Adhesions on floor of ret.; nail penetrating ret. was removed.
22	102.8	Her. 5 yr.	?	11,180	1,300 11.6	3,200 28.6	6,100 54.6	100 0.9	400 3.6	Adhesions on ventral floor of rumen.
23	101.6	Hol. 3 yr.	?	12,940	400 3.1	5,100 39.4	6,500 50.5	900 6.9	....	Adhesions in area of ret.; nail penetrating ret. removed.
24	103.3	Hol. 2 yr.	About 1 wk.	36,520	2,300 6.4	24,900 66.6	7,700 21.5	100 0.2	1,500 4.1	Adhesions of ret. to dia.; ret. held in higher than normal position.
25	103.0	Guern. 7 yr.	About 1 wk.	7,280	3,200 43.0	1,100 15.1	2,100 39.9	100 1.4	....	Adhesions under ret.; wire penetrating ret. removed.
26	101.0	Hol. 5 yr.	?	5,980	700 11.8	2,300 38.3	2,400 40.7	500 8.4	....	Some adhesions on ret.; no wires found penetrating.
27	101.5	Hol. 8 yr.	?	12,800	2,800 15.6	7,000 54.6	3,800 29.6	....	....	Adhesions, dia. to ret.; thickened area of reticular wall 4 in. in diameter.
28	102.0	Sh. 2 yr.	2 wk.	6,040	500 8.3	1,200 19.9	3,400 56.4	900 14.9	....	Adhesions of rumen to lateral abdominal wall; nail penetrating a fold was removed.
29	.....	Hol. 5 yr.	?	14,840	6,500 43.8	4,900 22.0	2,700 18.2	500 3.6	200 1.3	Adhesions of ret.; no wires found penetrating.
30	.....	Sh. 7 yr.	Losing wt. for 6 wk.	7,100	1,700 24.0	1,400 19.7	3,300 46.5	600 8.4	100 1.2	Wire penetrating ret. removed.
31	102.4	Hol. 2 yr.	?	13,720	2,000 14.5	4,800 35.0	6,100 44.4	600 4.3	200 1.4	Wire penetrating ret. removed.
32	101.6	Hol.	?	7,620	500 6.6	1,600 21.0	4,600 60.4	900 11.8	....	Adhesions between rumen and abdominal wall and in area of ret.; no foreign bodies in ret.
33	104.4	Sh. 3 yr.	?	11,760	4,000 34.2	1,300 11.2	6,300 53.6	100 0.8	....	Wire penetrating ret. removed.
34	101.2	Hol. 6 yr.	6 wk.	21,200	12,000 56.5	5,600 26.3	2,800 13.4	800 3.7	....	Wire penetrating wall of ret. removed.
35 (v.p.)	100.8	Swiss 6 yr.	?	10,900	4,400 40.4	5,500 50.5	900 8.2	....	100 0.9	Adhesions and abscess in area of ret. and liver.
36	101.7	Hol. 4 yr.	?	17,560	7,500 42.6	3,000 17.1	6,000 34.1	500 2.8	500 2.8	Wire penetrating ret. removed.
37	100.8	Hol. 7 yr.	?	8,240	2,700 32.7	1,600 19.3	2,500 30.0	400 4.7	1,000 13.3	Adhesions between ret. and dia.; nail penetrating ret. removed.
38	102.5	Hol. 4 yr.	6 wk. since calving.	11,080	1,700 15.3	3,600 32.5	5,400 48.7	300 2.5	....	Ret. adhered to abdominal floor; adhesions of spleen to rumen; loose metal in ret.
39	102.0	Guern. 1½ yr.	?	9,620	2,400 25.0	3,700 38.6	3,200 33.1	200 2.1	100 1.0	Adhesions between body wall, ret. and spleen; splenic abscesses.
40	101.0	Hol. 5 yr.	?	9,600	400 4.2	3,400 35.4	3,800 39.6	1,700 17.7	300 3.1	Adhesions between rumen and body wall; nail had penetrated ret.
41	102.0	Hol. 3 yr.	?	15,520	1,200 7.7	2,600 16.7	10,500 67.5	1,200 7.7	....	Granulation tissue involving floor of ret.; loose metal.
42 (v.p.)	101.5	Guern. 3 yr.	?	7,600	2,200 29.0	1,500 19.7	3,800 50.0	....	100 1.3	Adhesions between dia. and ret.; loose metal only.
43	102.0	Guern. 2 yr.	?	8,300	1,400 16.9	3,100 37.2	3,100 37.2	600 7.2	100 1.2	Adhesions of ret. to dia.; abscess on ret. surface; 2 nails penetrating ret. removed.

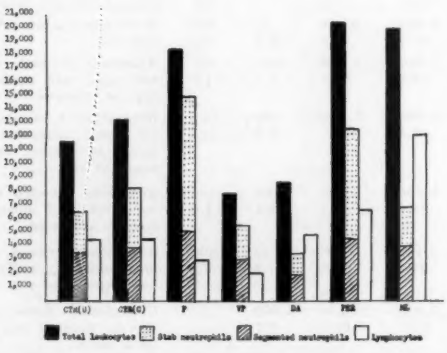
(Table 1 continued on next page)

TABLE 1 (Continued)—Hemograms of 50 Cattle with Relatively Uncomplicated Chronic Traumatic Reticulitis

Case No.	Temp. (F.)	Breed & age	History & duration	Leukocytes (total)	Neutrophils		Lymphocyte	Eosinophil	Monocyte	Laparotomy findings
					Stab	Seg.				
44	102.2	Hol. 7 yr.	Anorexia, 10 days	7,400	1,200 16.2	2,900 39.1	2,800 37.9	400 5.4	100 1.3	Adhesions of ret. to dia.; nail penetrating ret. removed.
45	102.4	Hol. 7 yr.	Anorexia, 10 days	15,650	4,700 30.0	2,500 16.0	6,600 42.1	1,700 10.8	100 0.7	Adhesions of ret. to dia.; wire removed from swelling on anterior face of ret.
46	101.8	Jersey ?	?	15,120	1,700 11.2	4,000 26.2	9,000 59.5	400 2.6	....	Adhesions between ret. and dia.
47	102.3	Guern. 8 yr.	1 wk.	8,920	2,100 23.3	600 6.7	5,200 58.3	700 7.7	300 3.4	Extensive adhesions between rumen, ret., and body wall; wire penetrating ret. removed.
48	100.6	Hol. 3 yr.	?	15,660	1,900 12.2	8,300 53.0	3,900 24.8	1,100 7.1	400 2.5	3 wires penetrating ret. removed.
49 (v.p.)	100.2	Hol. 7 yr.	2 wk.	8,640	1,000 12.0	5,100 59.1	2,000 23.1	500 5.8	....	Necropsied.
50	102.3	Swiss 2 yr.		8,500	1,900 22.2	500 5.8	4,800 56.5	1,300 15.3	....	Adhesions of rumen and ret.; abscess in spleen; enlargement in ruminoreticular fold thought due to foreign body.

scesses involving the liver, lungs, spleen, and various other locations including the joints and uterus. Other complicating conditions are present in some cases.

The animals with displaced abomasums, malignant lymphomas, and peritonitis not due to traumatic reticulitis are included to indicate the relative frequency of such cases and to illustrate the types of hemograms seen. However, due to the small number of these cases, no attempt is made to draw conclusions.



Graph 1—Comparison of differential and total leukocyte counts. Column at left of graph denotes leukocytes per cubic millimeter of blood. CTR (U) = chronic traumatic reticulitis, relatively uncomplicated; CTR(C) = chronic traumatic reticulitis, complicated; P = pericarditis; VP = vagal nerve paralysis; DA = displaced abomasum; PER = peritonitis, not due to traumatic reticulitis; and ML = malignant lymphoma.

RESULTS AND DISCUSSION

The normal total leukocyte count, reported by various authorities, varies considerably but there is general agreement that the neutrophils represent approximately 33 per cent and lymphocytes 56 per cent of the total, a relationship of 1.0 to 1.7.

The hemograms for normal adult cattle, at the Iowa State College clinic, have averaged approximately: total leukocytes, 9,800 ± 2,000 per cubic millimeter; neutrophils, 24 to 40 per cent; stab-segmented neutrophil ratio 1:5; lymphocytes, 55 to 65 per cent; eosinophils, 5 to 9 per cent; and monocytes, 0 to 2 per cent. These values compare favorably with averages of combined data of other workers.

In animals with the more complicated cases, such as pericarditis, the increase in total leukocytes and the differential count changes were much more marked (graph 1). This was of considerable value in indicating the extent of involvement.

Nearly 50 per cent of the animals with relatively uncomplicated traumatic reticulitis had a total leukocyte count below 10,000, which is well within the normal range. Therefore, in this type of case the total leukocyte count is of little value in diagnosis, a finding which concurs with that of other workers. However, a shift to the left was present in the differential count in a sufficient number of animals to

TABLE 2—Hemograms of 25 Cattle with Complicated Chronic Traumatic Reticulitis

Case No.	Temp. (F.)	Breed & age	Duration of illness	Leukocytes (total)	Neutrophils		Lymphocyte	Eosinophil	Monocyte	Laparotomy or necropsy findings
					Stab	Seg.				
1	101.0	Sh. 3 yr.	4 mo.	15,240	6,100* 40.4†	2,800 18.2	6,000 39.5	200 1.3	100 0.6	Lap.—adhesions of ret. to dia. Nec.—wire in abscess between ret. and dia.; multiple liver abscesses
2	.....	Guern. 4 yr.	?**	6,120	1,100 18.0	3,400 55.5	1,200 19.6	400 6.5	....	Lap.—adhesions, ret. to dia.; pleuritis.
3	102.0	Hol. 6 yr.	?	29,560	4,000 13.5	6,700 22.6	18,200 61.5	300 1.0	300 1.0	Lap.—adhesions between rumen, ret., & dia., & between spleen & rumen; hydroperitoneum; pericarditis. Nec.—as above; chronic general passive hyperemia; multiple pulmonary infarcts.
4	100.0	Her. 1 yr.	?	19,120	13,000 68.0	3,100 16.2	3,000 15.6	....	....	Nec.—traumatic reticulitis and splenitis; pyemia; focal necrotic suppurative hepatitis; chronic pancreatitis.
5	102.3	Her. 3 yr.	3 mo.	22,400	8,800 39.3	6,800 30.4	6,600 29.4	....	200 0.9	Nec.—traumatic reticulitis; compression of spinal cord at seventh thoracic vertebra by an abscess.
6	101.2	Angus 3 yr.	?	16,000	6,500 40.6	4,600 28.7	4,700 29.4	....	200 1.2	Nec.—perforated abomasal ulcer; wire penetrat-
7 (p.c.)	105.3	Hol. 4 yr.	1 mo.	16,700	8,500 51.0	3,500 21.0	4,400 26.2	200 1.2	100 0.6	Lap.—adhesions, ret. to body wall; abscess (5 " diam.) on ret. Nec.—as above; traumatic pericarditis—wire in pericardial sac.
8	102.0	Hol. 7 yr.	1 mo.	4,500	1,800 40.0	1,200 26.6	1,200 26.6	....	300 6.7	Nec.—traumatic reticulitis caused by wire; chronic focal suppurative hepatitis and pneumonitis; endocarditis; necrotic metritis.
9	.....	Guern. 3 yr.	?	4,180	800 19.3	500 12.1	2,500 60.4	100 2.4	200 4.8	Nec.—traumatic reticulitis; perireticular abscess; acute toxic hepatitis and nephritis; acute necrotic metritis.
10	101.0	Guern. 3 yr.	?	11,900	1,900 16.0	7,600 64.0	2,400 20.0	....	....	Nec.—traumatic reticulitis; large abscess involving ret., spleen, liver, and pericardium; fibrinous pleuritis and peritonitis; abomasal atony.
11	102.8	Guern. 5 yr.	3 wk.	5,500	1,400 25.4	300 5.5	3,800 69.0	....	....	Nec.—pulmonary abscess; adhesions between ret., dia., and lung; no tracts found.
12	104.6	Jersey 2 yr.	?	6,460	1,300 20.0	1,300 20.0	3,900 60.0	....	....	Nec.—peritonitis involving rumen, uterus, and body wall; traumatic reticulitis; toxemia; traumatic placentitis.
13	102.0	Hol. 1½ yr.	?	11,400	1,100 9.6	2,400 21.0	7,600 66.7	300 2.6	....	Lap.—adhesions, rumen to left abdominal wall; enlargement on ret.
14	102.0	Hol. 3 yr.	about 1 wk.	9,300	3,900 42.0	1,500 16.2	3,800 41.0	....	100 1.1	Nec.—traumatic reticulitis, tract containing wire extending from ret. into left lung; impaction and dilatation of abomasum; gangrenous pneumonia.

Seg. = segmented; ret. = reticulum; dia. = diaphragm; lap. = laparotomy; nec. = necropsy; p.c. = traumatic pericarditis; Hol. = Holstein-Friesian; Her. = Hereford; Sh. = Shorthorn; Guern. = Guernsey.

* Expressed as No. per cubic millimeter. † Expressed as percentage of total leukocytes.

** Question mark indicates that the condition was present for a minimum of one week.

(Table 2 continued on next page)

TABLE 2 (Cont.)—Hemograms of 25 Cattle with Complicated Chronic Traumatic Reticulitis

Case No.	Temp. (F.)	Breed & age	Duration of illness	Leukocytes (total)	Neutrophils		Lympho-cyte	Eosin-ophil	Mono-cyte	Laparotomy or necropsy findings
					Stab	Seg.				
15	101.8	Hol. 3 yr.	?	14,520	7,700 52.5	3,500 24.0	3,200 22.0	100 0.7	....	Lap.—adhesions on ret.; penetrating wire removed. Nec.—multiple abscesses and adhesions between ret. and dia.; pyemia; suppurative hepatitis; splenitis, and pneumonitis.
16	102.4	Sh. 2 yr.	?	16,710	4,300 25.6	6,400 38.2	5,400 32.1	100 0.7	500 2.9	Nec.—traumatic reticulitis; mediastinal abscess 18 cm. in diameter.
17 (p.c.)	103.0	Her. 5 yr.	2 mo.	22,050	18,600 84.0	1,500 6.8	1,900 8.6	....	....	Nec.—traumatic pericarditis and hepatitis; chronic suppurative pneumonia and hepatitis and pericarditis.
18	100.8	Angus 4 yr.	?	12,350	2,100 17.0	6,600 53.5	3,600 29.1	....	....	Nec.—traumatic reticulitis; acute suppurative peritonitis.
19 (p.c.)	102.4	Hol. 5 yr.	2 wk.	13,900	4,500 32.2	7,500 54.0	1,700 12.2	100 1.1	100 1.1	Lap.—adhesions between ret., liver, and dia.; metritis; wire in wall of ret. Nec.—traumatic reticulitis and pericarditis —wire in pericardial sac; suppurative metritis.
20	102.4	Hol. 4 yr.	?	15,220	1,800 11.8	9,300 61.0	4,200 26.6	....	....	Lap.—abscesses in spleen, rumen, and reticular walls; adhesions near spleen.
21 (p.c.)	.....	Hol. 3 yr.	?	19,170	10,400 52.8	5,700 28.9	3,400 17.2	200 1.1	....	Nec.—traumatic pericarditis; wire in dia.
22 (p.c.)	101.6	Hol. 7 yr.	?	19,660	8,500 43.1	6,900 35.0	4,100 20.8	....	100 0.5	Lap.—traumatic reticulitis; peritonitis; pericarditis; died later at home.
23	101.4	Guern. 2 yr.	?	8,000	2,200 27.5	3,600 45.0	2,000 25.0	100 1.2	100 1.2	Lap.—adhesions between ret., dia., and body wall; pericarditis (splashing sounds).
24	.....	Hol.	8 days	4,600	500 10.8	1,200 26.0	2,800 60.8	....	100 2.2	Lap.—fibrin on ret.; two wires penetrating. Nec.—many adhesions and abscesses in area of ret.; omasal atony; suppurative metritis.
25	.....	Guern. 2½ yr.	At least 2 mo.	7,560	2,500 33.1	1,500 19.9	3,200 42.5	300 3.9	....	Lap.—adhesions, ret. to dia.; nail penetrating ret. Nec.—tract extending from ret. through dia. and into heart; septic thrombus in ventricles.
			Mean	13,306	33.4	30.0	34.5	1.4	1.0	
			Percentage and range	(4,180–29,560)	(9.6–84.0)	(5.5–64.0)	(8.6–69.0)	(0–12.5)	(0–6.7)	

indicate its value in diagnosis. The mean total number of stab neutrophils was 2,956, while that of the segmented neutrophils was 3,738 per cubic millimeter, a ratio of 1:1.26. This is contrasted with the normal stab-segmented neutrophil ratio in adult cattle of 1:5. In only 5 animals was the stab-segmented neutrophil ratio normal. The stab neutrophils represented approximately 25 per cent of the total count; the segmented neutrophils about 30 per cent.

Analysis of the 50 cases of relatively uncomplicated chronic traumatic reticulitis shows that the normal relationship of 1.7 lymphocytes to 1.0 neutrophils was reversed in 38 per cent. Actually, 65.3 per cent showed a majority of neutrophils. The lymphocytes averaged 40 per cent as con-

TABLE 3—Summary of Hemograms of 50 Animals with Relatively Uncomplicated Chronic Traumatic Reticulitis

Item	Leukocytes (total)	Neutrophils			Lymphocyte	Eosinophil	Monocyte
		Total	Stab	Seg.			
Mean	11,799	53.9%	24.6%	29.3%	39.6%	5.0%	1.2%
Range	4,100–36,520	24.4–90.0%	3.0–56.5%	2.4–66.6%	8.2–67.5%	0.0–67.5%	0.0–13.3%
Median	10,790	6,714–4,869*	2,956–2,698*	3,738–3,631*	4,406–2,154*	.....	.....
Actual range of differential	.....	1,700–27,200	200–12,800	100–24,900	900–10,500	.....	.....
Median of differential	.....	5,400	2,100	3,050	3,850	.....	.....

* Standard deviation.

TABLE 4—Hemograms of Animals with Peritonitis Not Due to Reticulitis

Case No.	Temp. (F.)	Breed & age	Duration of illness	Leukocytes (total)	Neutrophils		Lymphocyte	Eosinophil	Monocyte	Necropsy findings
					Stab	Seg.				
1	103.6	Sh. 3 yr.	?*	9,650	2,400† 25.2**	3,300 34.4	3,900 40.4	....	....	Perforating ulcer of colon; acute diffuse suppurative peritonitis (ADSP).
2	103.3	Hol. 4 yr.	?	26,120	14,900 56.2	5,400 20.4	5,800 22.2	....	200 0.8	Perforation of anterior dorsal wall of vagina; perforation of spiral colon; ADSP.
3	101.2	Hol. 4 yr.	2 mo.	9,740	2,000 20.1	2,100 21.1	4,800 48.2	1,100 11.0	....	Multiple abscesses throughout abdominal cavity and viscera.
4	103.0	Hol. 2 yr.	?	40,840	16,900 41.2	11,800 29.6	9,900 24.1	1,500 3.5	700 1.6	Abscess (2½ ft. diam.) ventral to rumen; many adhesions; ADSP.
5	101.3	Sh. 2 yr.	?	15,300	8,800 57.5	1,000 6.5	4,700 30.7	600 3.9	200 1.3	Lacerations of vagina; perivaginal phlegmon; hemoperitoneum; ADSP.

Seg. = segmented; Sh. = Shorthorn; Hol. = Holstein-Friesian.
* Question mark indicates that the condition was present for a minimum of one week.
† Expressed as No. per cubic millimeter. ** Expressed as percentage of total leukocytes.

TABLE 5—Hemograms of Animals with Malignant Lymphoma

Case No.	Temp. (F.)	Breed & age	History & duration	Leukocytes (total)	Neutrophils		Lymphocyte	Eosinophil	Monocyte	Necropsy findings
					Stab	Seg.				
1	101.5	Angus 7 yr.	?*	18,060	3,200† 17.7**	9,000 50.0	5,500 30.2	100 0.5	200 1.1	Malignant lymphoma (ML); lymph nodes throughout body involved.
2	101.4	Sh. 6 yr.	1 wk.	3,800	600 15.7	1,200 31.6	1,900 50.0	....	100 2.7	ML; multiple tumor foci in lungs.
3	.....	Angus 5 yr.	?	18,040	6,000 33.1	2,400 13.3	8,000 44.2	1,200 6.6	400 2.2	ML; massive involvement of nodes of pelvic and perirenal region; infiltration into vertebral canal.
4	101.0	Hol. 6 yr.	?	41,440	2,000 4.8	400 0.9	38,500 93.0	....	500 1.2	ML; marked involvement of all lymph nodes and heart, liver, spleen, and uterus.
5	101.5	Jersey 5 yr.	?	14,280	1,000 7.1	3,200 22.2	9,500 66.5	500 3.5	100 0.7	ML; iliac, mesenteric, and cervical nodes enlarged; metastasis to liver, kidneys, and uterus.
6	101.0	Hol. 5 yr.	Progressive emaciation	24,640	2,000 8.1	1,100 4.6	20,800 84.0	400 1.6	300 1.3	ML of abomasum; metastasis to lymph nodes, genital tract, and heart.

Seg. = segmented; Sh. = Shorthorn; Hol. = Holstein-Friesian.
* Question mark indicates that the condition was present for a minimum of one week.
† Expressed as No. per cubic millimeter. ** Expressed as percentage of total leukocytes.

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trasted to the normal of about 56 per cent. However, in 50 per cent of the cases, the number of lymphocytes was over 4,000 per cubic millimeter, which is within the normal range; in other cases, the lymphocytes were reduced in total number. Thus the reversal of the lymphocyte-neutrophil ratio is usually not due to a lymphopenia but to neutrophilia, with a marked increase in the proportion of immature forms.

In animals with chronic traumatic reticulitis, body temperature findings are of little significance, since little or no elevation occurs in the majority of cases; also, the erythrocyte count and hemoglobin determinations are not of diagnostic value.

Data on peritonitis (not due to traumatic reticulitis), displaced abomasum, and malignant lymphoma are presented (tables 4–6) to give a comparative picture of these disease syndromes in cattle. Five animals with vagal nerve paralysis (cases 10, 11, 35, 42, 49) are included under the relatively uncomplicated chronic traumatic reticulitis group (table 1), and 5 with pericarditis (cases 7, 17, 19, 21, 22) are included in the complicated group (table 2). Although the reader may separate these cases in his own mind to obtain a typical blood picture for these two syndromes, the number of cases is insufficient to justify any statistical summation.

Six animals (cases 8, 10, 11, 35, 42, 49) with relatively uncomplicated chronic traumatic reticulitis were necropsied; the last 5 had a concurrent vagal paralysis (table 1). Others may have died later at home, since follow-up reports are not available.

The value of the standard deviation (SD) as a measure of dispersion is lessened when some of the figures are not distributed symmetrically around the mean. It should also be remembered that a large deviation makes the mean representative of the central tendency in a group. Several animals in this study have what appear to be atypical values for one or two blood elements, and this affects the mean correspondingly. The following statements refer only to the

TABLE 6—Hemograms of Animals with Displaced Abomasums

Case No.	Temp. (F.)	Breed & age	Duration of illness	Leukocytes (total)	Neutrophils		Lympho-cyte	Eosin-ophil	Mono-cyte	Laparotomy or necropsy findings
					Stab	Seg.				
1	102.0	Hol. 3 yr.	3 wk.	5,600	1,300* 23.2†	900 16.2	3,200 57.0	100 1.8	100 1.8	Nec.—abomasum displaced under and to left of rumen; pulmonary emphysema.
2	101.2	Swiss 6 yr.	?**	8,400	1,300 15.5	2,300 27.3	4,200 50.0	400 4.8	200 2.4	Lap.—abomasum high on left side; replaced.
3	100.6	Hol. 5 yr.	?	12,880	1,200 9.2	4,200 32.2	7,200 56.3	300 2.3	....	Lap.—abomasum displaced backward and to left; replaced; small intestines near left paralumbar fossa.
4	101.8	Swiss 6 yr.	?	7,000	1,100 15.7	1,500 21.4	4,000 57.0	300 4.3	100 1.4	Lap.—abomasum entirely displaced to left side; replaced.
5	101.2	Hol. 3 yr.	anorexia, 1 wk.	8,980	400 4.6	2,600 29.0	4,500 50.2	1,200 13.3	200 2.3	Lap.—omentum along left rumen wall; flaccid abomasum displaced to left between rumen floor and pregnant uterus.
6	102.4	Guern. 2 yr.	?	10,720	1,900 17.7	4,100 38.1	4,200 39.1	300 2.8	200 1.8	Lap.—displaced abomasum; replaced
7	102.9	Hol. 4 yr.	?	5,420	400 7.4	900 16.6	4,100 76.0	....	....	Lap.—abomasum displaced entirely to left side; about 4″ below vertebral column; flaccid and atonic; replaced.
8	101.0	Her. 1 yr.	?	9,640	1,000 10.4	600 6.2	7,000 72.5	800 8.3	200 2.1	Lap.—flaccid abomasum under distended rumen; replaced.
9	101.6	Guern. 5 yr.	?	9,980	4,400 45.2	1,600 16.2	3,800 38.2	100 0.9	....	Lap.—omentum and part of abomasum on left side of rumen; replaced.

Seg. = segmented; nec. = necropsy; lap. = laparotomy; Hol. = Holstein-Friesian; Guern. = Guernsey; Her. = Hereford.

* Expressed as No. per cubic millimeter. † Expressed as percentage of total leukocytes.

** Question mark indicates that the condition was present for a minimum of one week.

group of relatively uncomplicated chronic traumatic reticulitis cases (table 1).

The SD for the total leukocyte count was 5,846/cmm. By omitting the square of the deviations from the mean of cases 3, 17, and 24 from the calculations for the SD, the SD will be 4,066/cmm.*

In computing the mean for the neutrophils, if cases 17, 24, and 34 are omitted, the mean is 5,755 instead of 6,714/cmm. By omitting the values for the deviation squared of cases 17, 24, and 34 in computing the SD for the neutrophils, the SD is 3,127 instead of 4,869/cmm.*

If, in computing the mean values for the stab and segmented neutrophils, one omits case 17 (in stab calculations) and case 24 (in segmented calculations), the means are 2,734 instead of 2,956/cmm. for the stab neutrophils and 3,306 instead of 3,738/cmm. for the filament neutrophils. By omitting the value for the deviation squared of case 17 in computing the SD for the stab neutrophils, the SD is 2,243 instead of 2,698/cmm. Similarly, by omitting the deviation squared value for case 24 in computing the SD for the segmented neutrophils, the SD is 2,077 instead of 3,631/cmm.* (The deviation squared value of the segmented neutrophils of case 24 is more than twice as great as the total of the squared values of the other 49 cases.)

It is felt that a routine blood examination in suspected cases of chronic traumatic reticulitis offers considerable value as a diagnostic and prognostic aid. The most important single feature is the increased number of neutrophils in proportion to the lymphocytes. Thus, while a total white blood cell count may be sufficient in acute cases, a differential count should always be included as an aid to diagnosis in chronic cases. It has been shown that the stab-segmented neutrophil ratio is usually markedly altered, with a great increase in the proportion of immature neutrophils (shift to the left). The hemogram, however, is not always conclusive and should only be considered as supportive evidence.

* The mean deviation values used in computing this new SD were the same as those calculated from the original mean. It is realized that in order to get a new valid SD, one should figure a new mean and thus compute a new set of mean deviation figures, omitting the case in question. The new figures are presented to give an approximate illustration of how a few wide deviations from the central tendency can markedly alter the "usual" mean and SD which would be seen in the majority of cases.

SUMMARY AND CONCLUSIONS

1) Of 50 cattle with relatively uncomplicated chronic traumatic reticulitis, 65.3 per cent showed more neutrophils than lymphocytes. In 38 per cent, there was a complete reversal of the normal lymphocyte-neutrophil ratio of 1.7:1.0.

2) The ratio of the stab neutrophils to the segmented neutrophils in affected animals was 1:1.26, whereas in normal animals the ratio is 1:5. In only 5 cases of 50 was the stab-segmented neutrophil ratio normal. The shift to the left indicates the response of the body to a continuing source of infection and irritation.

3) Nearly 50 per cent of the above 50 animals had a total leukocyte count below 10,000 per cubic millimeter.

4) Of 25 animals with complicated traumatic reticulitis, 64 per cent showed a complete reversal of the normal lymphocyte-neutrophil ratio. The increase in total leukocytes was more marked in animals in this group than in the uncomplicated group.

5) A blood examination, including the total leukocyte count and differential count, is of value in the diagnosis and prognosis of chronic traumatic reticulitis. Even though one feature may be within normal limits at a particular time, it is probable that a differential count will disclose a significant disturbance.

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Experimental Stenosis of the Pulmonary Artery in Calves

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DECOMPENSATION of the right side of the heart is one of the characteristics of the disease syndrome in cattle known as "brisket disease." Primary lung lesions, congestive fibrosis of the liver, and extensive generalized edema are found at necropsy.[27]

This investigation was undertaken to determine if stenosis of the pulmonary artery would produce cardiac and hepatic changes similar to those found in calves with clinical cases of brisket disease.

REVIEW OF LITERATURE

Surgical methods to decrease arterial diameters with metal bands were initiated by Halsted[13] in 1905. He later applied metal[14] and tape[15] to produce stenoses of arteries. Holman and Beck[17] and Gerbode and Selzer[10] determined that fatal erosion frequently occurred when the pulmonary artery was partially occluded. Fascia was considered unreliable for ligation.[21] Various other methods and devices used in earlier works on large arteries have been discussed by Pearse.[25]

Some workers produced hypertrophy of the right side of the heart, but it was not accompanied by dilatation of the right ventricle.[16, 28]

The most recent methods used to produce hypertrophy of the right ventricle were (1) silk sutures placed across the midportion of the conus arteriosus;[1, 2] (2) concentrated nitric acid painted on the pulmonary valve cusps and sinus of Valsalva;[20] (3) partial closure of the pulmonic valve cusps with sutures;[19, 29] and (4) constriction of the pulmonary artery with laminated cellophane bands.[5]

Haggart and Walker[12] determined that the pulmonary artery had to be 52 to 66 per cent occluded before significant circulatory changes appeared. At least 60 per cent constriction was necessary before right atrial or aortic pressures were altered[31] and a detectable change in heart work was produced.[11] If the stenosis was greater than 60 to 70 per cent, death occurred within one to three days.[3] Donald and Kirkin[5] observed that a 63 per cent reduction in vessel lumen size had to be present before right ventricular hypertension was evident; one exception was a 40 per cent reduction.

Liebow *et al.*[22] produced cardiac hypertrophy and hepatic passive congestion when the left pulmonary artery was completely transected and the right pulmonary branch was ligated to one third of its original diameter. According to Hufnagel *et al.*,[18] the most successful method used produced a 50 per cent stenosis. This occluded 75 per cent of the cross-sectional area of the pulmonary artery. When the stenosis was 60 to 80 per cent, fatal results occurred within 24 to 72 hours.

Biopsy methods for liver study in cattle were first used by Garner,[9] who preferred to place the animal in left lateral recumbency. Loosemore and Alcroft[23] believed that cattle should be biopsied in the standing position, because the liver was displaced in recumbency. Seghetti and Marsh[30] and Bone[4] used similar techniques without fatality. According to Udall *et al.*,[32] the procedure was facilitated when the animal's back was arched to bring the liver in contact with the abdominal wall. Whitehair *et al.*[34] reported favorable results in calves when samples were obtained by laparotomy.

MATERIALS AND METHODS

In this study, 9 dairy calves of mixed breed, 4 to 8 months old, were used. One of these calves was used as the control animal. The interval from the time of occlusion of the pulmonary artery to necropsy was 91 to 122 days.

The initial anesthetic was a 2.5 per cent aqueous solution of thiamylal sodium* and, in some cases, pentobarbital sodium† administered intravenously. Anesthesia was maintained by ether inhalation with a Foregger gas machine.

The ligation procedure was performed with the animal in right lateral recumbency. A 9-inch incision was made over the left fourth rib, and approximately 6 inches of the rib was resected. The pericardium was incised over the conus arteriosus and the pulmonary artery loosened by blunt dissection. A double strand of ⅛-inch umbilical tape was used for all ligations with the exception of calf 54R2, where No. 4 braided nylon was used. In all animals, the artery was ligated to approximately 50 per cent of its original diameter.

After ligation, the thorax was closed by standard technique. The control calf, 54R23, was operated on in the same manner, with exception of the ligation procedure.

The liver of each animal was biopsied three days before ligation and at intervals five to 14 days after ligation. Each biopsy was performed under local anesthesia on the right side of the animal (fig. 1) with an instrument similar to the one described by Seghetti and Marsh.[30] A total of 44 biopsies was completed.

From a thesis submitted to the Graduate School, College of Veterinary Medicine, Colorado State University, Fort Collins, in partial fulfillment of the requirements for the M.S. degree.

* Surital sodium, produced by Parke, Davis & Co., Detroit, Mich.

† Nembutal sodium, produced by Abbott Laboratories, North Chicago, Ill.

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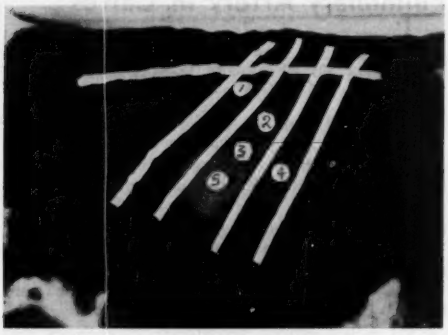

Fig. 1—Location of liver biopsy punctures, calf 54R21.

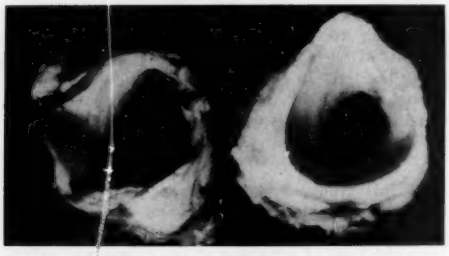

Fig. 2—Cross-section of pulmonary artery of normal calf (left) and ligated calf 54R22.

Blood samples were taken from each calf at the time of the liver biopsy for a period of 60 days. The total erythrocyte count, the total and differential leukocyte count, and the hemoglobin and hematocrit values were determined from each sample. The serum protein, sodium, and potassium values were determined at intervals following ligation. Serum protein values were obtained by the copper sulfate specific gravity method. Sodium and potassium values were determined by the flame photometer method.

Gross examination of each calf was made at necropsy. The weights of the heart, liver, spleen, and kidney were recorded. The hearts and heart weights of the calves with pulmonary artery stenosis were compared with those of normal animals. The diameter at the ligation site of each artery was compared with the same site of a normal pulmonary artery (fig. 2). An asymmetric "t" test was used to determine if a significant difference existed between the paired heart weights of the ligated and normal animals.

The tissues from each liver biopsy, and the liver and spleen taken at necropsy, were sectioned and prepared by standard techniques. Each slide was examined for pathological alteration.

RESULTS

The control calf and 4 treated calves survived the 90-day period after the 50 per cent ligation of the pulmonary artery; 4 calves died from heart failure or pulmonary complications, or both (table 1). An abnormal cardiac bruit and jugular vein distention developed immediately after the ligature was applied. The distention disappeared within 18 hours.

The blood counts, hemoglobin values, and hematocrit values were within normal limits throughout the study. The sodium, potassium, and serum protein levels were also within normal limits.

All liver biopsy samples from the calves with stenoses were normal with the exception of 2 animals. At 34 days after ligation, calf 54R22 developed a marked passive congestion of the liver which continued

TABLE 1—Necropsy Findings on 8 Calves After Partial Occlusion of the Pulmonary Artery

Animal (No.)	Experimental period (days)	Cause of death	Necropsy findings
54R1	122	Killed	Adhesions of left lung to pleura; visceral organs normal.
54R2	120	Killed	Adhesions of left lung to pleura; right ventricle slightly hypertrophied; visceral organs normal.
54R3	14	Septicemia and pneumonia	Marked fibinous pneumonia and pleuritis; pericardial and reticular abscesses; visceral organs normal.
54R4	6	Anoxia and right heart failure	Slight dilatation of right ventricle; anemic infarcts in right lung; visceral organs normal.
54R5	6	Pneumonia and right heart failure	Passive congestion in liver; fibrinous pneumonia and pericardial adhesions; heart and spleen normal.
54R6	2 hours	Heart failure	Excessive constriction of pulmonary artery; visceral organs normal.
54R21	100	Killed	Right ventricle hypertrophied; slight pericardial and pleural adhesions; liver and spleen normal.
54R22	97	Killed	Right ventricle hypertrophied; passive congestion of liver; spleen normal.
54R23*	91	Killed	Heart and lungs normal; visceral organs normal.

* Control animal.

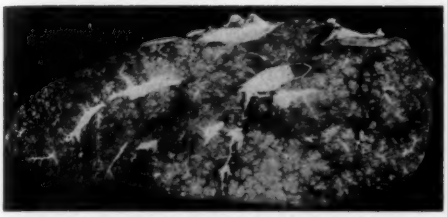

Fig. 3—Cut section of liver from calf 54R22, showing passive congestion. The normal tissue is light colored.

to increase until the animal was killed. Calf 54R21 showed slight passive congestion of the liver 65 days after the ligation.

At necropsy, the liver of calf 54R22 was nodular, the capsule thickened, and the tissue was cut with resistance. On the cut surface, a dark red reticulation was superimposed on a light colored, normal tissue (fig. 3). Microscopically, the centrilobular zones contained atrophied hepatic cells. Chronic passive congestion was indicated by dilatation of the central veins and sinusoids. Early fibrosis was evident in these areas (fig. 4, 5).

The histopathological changes in the liver of calf 54R21 showed a slight hyperemia in the central zones of the lobules. The central veins and surrounding sinusoids were slightly dilated.

The hearts of calves 54R21 and 54R22 showed gross hypertrophy and dilatation. The heart weights of the calves with pulmonary artery stenosis were significantly heavier than the heart weights of the normal calves ($P>.05$).

DISCUSSION

Two of the pathological changes found in brisket disease of cattle were produced by 50 per cent stenosis of the pulmonary artery. These changes were hypertrophy and dilatation of the right ventricle of the heart and passive congestion of the liver. The pathogenesis of the changes appeared to be as follows: Stenosis of the pulmonary artery produced an increase in resistance to the flow of blood from the right ventricle. The work load of the ventricle was increased enough to cause hypertrophy and dilatation. This enlargement created a valvular insufficiency and venous congestion, indicating that decompensation of the heart had occurred. Circulation of blood

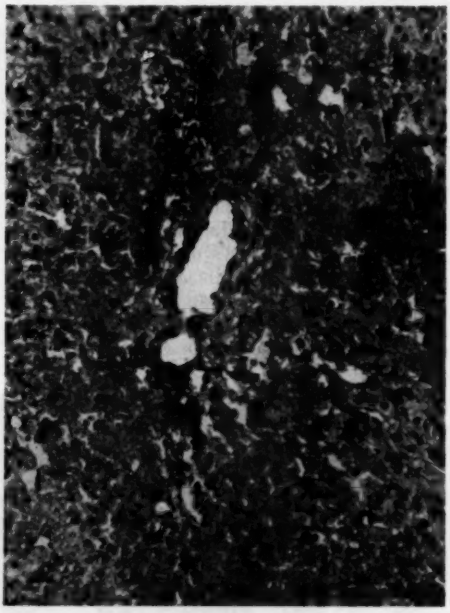

Fig. 4—Liver from calf 54R22, showing passive congestion with atrophy of hepatic cords in the central zone of a lobule. Fibrosis is beginning. H & E stain; x 140.

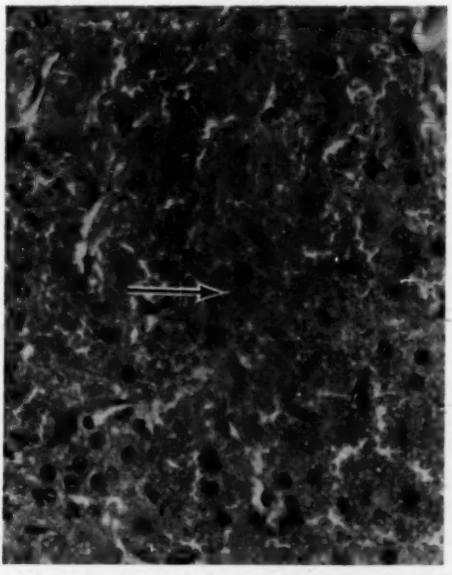

Fig. 5—Liver from calf 54R22, showing passive congestion and atrophic hepatic cords (arrow). H & E stain; x 280.

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through the liver was slowed to the extent that passive congestion developed. If the time interval from ligation to necropsy had been longer, other changes such as edema, hydrothorax, and ascites might also have developed.

Chronic pulmonary disease can cause hypertrophy and dilatation of the right ventricle of the heart.[8] The presence of various pulmonary diseases has been reported in clinical cases of "high mountain sickness" (brisket disease) of cattle.[26] Therefore, an interference in pulmonary circulation might exist early in the clinical case and cause the cardiac decomposition.

of the patients with chronic congestive heart failure.

Liver biopsy proved to be of considerable value in detecting the appearance of passive congestion. Its value as a diagnostic procedure in man has been shown.[32,33] In agreement with Udall *et al.*,[31] the arching of the back of the animal to bring the liver in contact with the right side during the procedure proved advantageous. As determined by Loosemore and Alcroft,[23] excessive negative pressure within the syringe when taking the biopsy sample damaged the tissue sections.

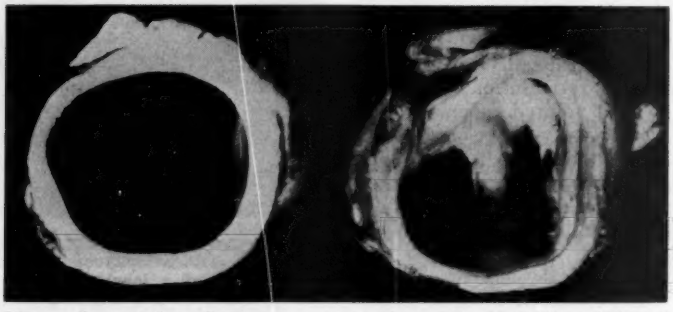

Fig. 6—Cross-section of pulmonary artery of normal calf (left) and ligated calf 54R2, showing effects of suture erosion of vessel wall without fatality.

As observed in this study, cardiac failure can lead to hepatic congestion.

The degree of vessel constriction used in this study agrees with Hufnagel *et al.*,[18] who found that the most successful procedure produced a 50 per cent stenosis of the pulmonary artery. The deaths of 3 calves in this study were attributed in part to cardiac failure from excessive constriction of the vessel.

The use of umbilical tape did not cause fatal erosion of the vessel as described by Holman and Beck,[17] but fibrotic tissue was formed around the tape in each case. The nylon ligature severed the vessel wall but did not cause fatal erosion (fig. 6).

Since no abnormal alterations appeared in the blood cell counts or the hemoglobin, hematocrit, sodium, potassium, and serum protein values, a longer interval apparently must be required to produce significant changes. In human patients, low level plasma proteins were frequently found in cases of cardiac failure.[6] Felder [7] reported a decrease in plasma proteins in 29 per cent

SUMMARY

Stenosis of the pulmonary artery was produced in 8 dairy calves by constricting the vessel to approximately 50 per cent of its original diameter. Four animals survived, 3 animals died of complicated heart failure, and 1 animal died from infectious processes.

Marked passive congestion of the liver developed in 1 of the surviving animals 34 days after ligation. Early centrilobular fibrosis was evident at necropsy. Another calf developed slight hepatic passive congestion 65 days after ligation.

An asymmetric "t" test showed that the heart weights of the 4 animals with stenosis were significantly heavier than the heart weights of the 4 comparable normal animals.

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Electrophoretic Studies of Bovine Serum. I. Changes in the Serum Proteins After Splenectomy

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ANIMALS that have been injured experimentally and subjected to shock [5, 6, 12–14] and persons who have undergone surgery [3] show marked alterations in their serum proteins. Generally, there are decreases in the relative and absolute concentrations of albumin, whereas the concentrations of globulins increase.

In investigations dealing with experimental infections of anaplasmois in calves, it became necessary to remove spleens prior to infection in order to increase susceptibility.[10] Serum proteins were analyzed during the study and results are presented on the changes that occurred after splenectomy and prior to experimental infection with this disease.

MATERIALS AND METHODS

Cattle.—During the course of this investigation of the effects of splenectomy on serum proteins, 23 dairy calves of mixed breeds, 3 weeks to 6 months old, were obtained and studied. They were housed in pens in a closed-type screened barn. Calves were not isolated individually within the unit. Young calves were fed commercial milk supplement containing 25 per cent protein for approximately four weeks after birth and then placed on a 16 per cent protein dairy feed with grass hay. Splenectomy was performed on young animals at varying time intervals after they had been on dairy feed for approximately 14 days. No complications were observed. Antimicrobial therapeutic agents were not used after surgery. Older animals had been on dairy feed and hay for approximately four months prior to splenectomy.

Blood Smears.—Air-dried blood smears were stained with Giemsa's stain to detect possible natural infection with anaplasmosis or eperythrozoonosis.[4]

Determination of Serum Proteins.—Blood collected from the calves was allowed to clot, and the serum was decanted and clarified by centrifugation. Samples were separated by paper strip electrophoresis using barbital buffer of pH 8.6 and 0.075 ionic strength. A current of 0.6 ma. per strip was applied for 17 hours. At this time the strips were dried, fixed, and stained with bromphenol blue according to conventional procedures.[1] Each strip was scanned photometrically for the determination of the relative concentrations of albumin, *alpha*, *beta*, and *gamma* globulins. In order to convert these relative concentrations of serum fractions to absolute values (Gm./100 ml. of serum), protein nitrogen determinations were also made on all serum samples. Trichloroacetic acid precipitates of the proteins were digested with sulfuric acid and the converted ammonia determined spectrophotometrically by direct nesslerization.

RESULTS

Splenectomized Calves.—A total of 17 calves were studied in this group. In general, after removal of the spleen the absolute values of *gamma* globulins (GG), total globulins (TG), and total serum proteins (TSP) decreased. Less globulins on a weight basis (Gm./100 ml. of serum) were present in the serum. The albumin/globulin (A/G) ratio also declined, showing that a smaller proportion of albumin to globulin was present. The decreases in the values of GG, TG, and TSP were temporary and persisted for approximately one month, when they returned to values essentially similar to those prior to surgery. The A/G ratios, however, did not return to the preoperative levels until approximately two months after splenectomy. After this period, there was a slight rise in the A/G ratio above the preoperative values. The A/G ratio values returned to preoperative levels within three months after surgery and remained essentially static until the termination of the experiment at four months.

In 7 calves, a transient increase in the absolute concentration of *alpha* globulin was observed. This increase was first noticed approximately one week after surgery and persisted for one week, when the concentration of *alpha* globulin returned to a normal level.[9]

Data showing the mean values for the various serum proteins and A/G ratios in 17 splenectomized calves are given (table 1). The values of the serum proteins were

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within normal limits, as reported by Pierce,[9] prior to and after recovery from surgery.

Normal Calves.—In this group, 6 calves were studied. Mean values (table 2) show the changes that occurred in the serum proteins of these animals over a period of 110 days. The over-all differences, as compared with the splenectomized calves, are apparent. All serum protein values were well within the normal range and severe alterations in the serum proteins, as were observed in the splenectomized calves, did not occur.

Major fluctuations in the A/G ratios appeared in the series of serum protein analyses between individual calves within each group before calculations of the means of all animals were made. The A/G ratio did not represent a quantitative value, since it only denoted the relative proportions of albumin to globulins which were calculated from percentage values of these components. Consequently, a true picture of the serum proteins was not indicated. Absolute weights of the proteins based on quantitative figures should be evaluated as they have been in the present study.

DISCUSSION

When splenectomy was performed, there were decreases in the TSP, GG, and TG based on grams per 100 ml. of serum. These decreases persisted for approximately one month after surgery when TSP, GG, and TG returned to values essentially similar to those prior to splenectomy. In a number of cases, a transient increase in the *alpha* globulin was noticed during the second week following surgery. This increase persisted for approximately one week. *Beta* globulin levels did not fluctuate appreciably during the entire period of study. The present findings were compared with reports dealing with studies on the serum and plasma proteins in subjects undergoing surgery[3] or experimental injury and shock.[5, 6, 12–14] It was found that, in general, globulins increased, whereas albumin and total serum proteins decreased. None of the above studies was concerned with splenic surgery or injury.

When recovery patterns were followed,[3] preoperative levels of the serum proteins returned after four days. The differences in the results obtained in the recovery rates of the serum proteins and the fact that globulins decreased after splenectomy in the calves studied in this report can not be fully explained. It seems plausible to assume that, since splenic tissues are responsible for the production of plasma proteins,[7, 8, 11] at least one month must elapse for nonsplenic lymphoid tissues to take over the task, in part, in the production of plasma proteins. It is of particular interest that the production of antibody proteins was not inhibited in splenectomized persons who had been immunized with tetanus toxoid,[7] whereas inhibition in antibody protein production occurred in splenectomized cattle infected with anaplasmosis.[11]

TABLE 1—Electrophoretic Analyses of Serums from 17 Splenectomized Dairy Calves

Days (bleeding intervals)	Mean serum protein values					
			Globulins			
	TSP	A/G	*Alpha*	*Beta*	*Gamma*	Total
0	7.06	0.78	1.10	0.98	1.34	3.42
1	SPLENECTOMIZED					
3	6.22	0.48	1.07	0.95	1.20	3.22
7	5.74	0.47	1.26	0.93	0.92	3.11
17	5.07	0.55	1.17	0.92	0.65	2.74
24	5.82	0.58	1.06	0.93	0.89	2.88
29	6.61	0.53	1.14	0.92	1.20	3.26
31	6.93	0.62	1.16	0.99	1.35	3.50
38	7.13	0.72	1.04	0.94	1.39	3.37
47	6.90	0.74	1.15	0.99	1.28	3.42
59	6.94	0.81	1.16	0.97	1.36	3.49
65	7.21	0.89	1.06	1.00	1.32	3.38
72	7.15	1.07	1.00	0.96	1.40	3.36
79	6.99	1.17	1.08	0.99	1.33	3.40
85	6.89	0.96	1.07	0.94	1.33	3.34
92	7.00	0.85	1.14	0.93	1.30	3.37
98	7.19	0.80	1.09	0.96	1.37	3.42
113	7.10	0.76	1.17	0.94	1.33	3.44
120	7.21	0.76	1.14	0.91	1.40	3.45

TSP = total serum protein as grams per 100 ml. of serum; A/G = albumin/globulin ratio; globulins = grams per 100 ml. of serum.

TABLE 2—Electrophoretic Analyses of Serums from 6 Normal Dairy Calves

Days (bleeding intervals)	Mean serum protein values					
			Globulins			
	TSP	A/G	*Alpha*	*Beta*	*Gamma*	Total
0	6.79	0.80	0.98	1.12	1.47	3.57
5	6.93	0.77	1.15	1.10	1.37	3.62
12	6.84	0.78	1.16	1.07	1.39	3.62
19	6.67	0.81	0.95	1.07	1.50	3.52
26	6.57	0.76	0.93	1.09	1.46	3.48
33	6.72	0.82	0.91	1.11	1.40	3.42
40	6.90	0.81	1.00	1.08	1.43	3.51
47	6.80	0.79	1.04	1.12	1.38	3.54
54	6.88	0.76	0.92	1.14	1.49	3.55
61	6.62	0.84	1.16	1.07	1.38	3.61
68	6.98	0.77	1.14	1.13	1.39	3.66
75	6.92	0.78	1.14	1.06	1.35	3.55
82	6.59	0.80	1.08	1.08	1.36	3.52
89	7.02	0.79	1.13	1.11	1.36	3.60
96	6.74	0.82	0.93	1.10	1.41	3.44
103	6.53	0.86	0.98	1.14	1.40	3.52
110	6.66	0.77	1.12	1.13	1.45	3.70

TSP = total serum protein as grams per 100 ml. of serum; A/G = albumin/globulin ratio; globulins = grams per 100 ml. of serum.

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The increase in the A/G ratios above the presurgical levels, after two months had passed since splenectomy, may have been of importance in maintaining the osmotic pressure balance of the serum proteins. However, the absolute weights of these proteins had returned to normal in this time after the initial decreases. Possibly, it takes much longer than realized to recover osmotic pressure balance after surgery, although this is not evident from the electrophoretic analyses and total serum protein studies. Since albumin contributes more to osmotic pressure than do the globulins,[2] a compensatory increase in this fraction may still be required after surgical shock, loss of blood, and recovery. The possibility also exists that hypertrophic nonsplenic lymphoid tissues produce this increased proportion of albumin to globulin.

SUMMARY

The effects of splenectomy on the serum proteins of dairy calves were studied by paper electrophoretic and serum protein techniques.

Removal of the spleen caused a decrease in the total serum proteins, *gamma* globulin, and total globulins which persisted for one month after surgery when these values returned to normal.

The albumin/globulin ratio decreased after splenectomy and regained its preoperative value after two months. At this time, the ratio increased but returned to normal values in an additional month.

Alpha globulin showed a slight rise during the second week after surgery, whereas *beta* globulin levels did not fluctuate appreciably during the study.

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Electrocardiograms of Normal, Lactating Dairy Cows

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ELECTROCARDIOGRAPHIC STUDIES on the normal, lactating dairy cow have been reported only occasionally in veterinary literature. This study was attempted in the hope of adding information on the appearance of the waves of the electrocardiogram as well as the intervals of the various wave complexes of the normal lactating dairy cow.

REVIEW OF LITERATURE

Alfredson and Sykes[1] reported on a series of three tracings from each of 97 dairy animals, ranging in age from 5 months to 12 years, using the three standard limb leads. They reported on duration of intervals and systolic indexes, and proposed a classification based upon the appearance of the QRS in lead II. Platner *et al.*[9] reported on intervals, systolic indexes and heart rates in 10 dairy cows ranging from 3 to 9 years old using the three standard limb leads. Sellers *et al.*[10] reported less variability in tracings from animal to animal was obtained with a bipolar chest lead than with the three standard bipolar limb leads used on cattle.

MATERIALS AND METHODS

A series of three electrocardiograms, secured over a 15-day period, was made on each of 45 normal, lactating dairy cows ranging in age from 2 to 12 years. The group consisted of 25 Holstein-Friesian, 13 Brown Swiss, and 7 Ayrshire cows, and they ranged from nonpregnant to seven months pregnant. The heart of each cow was auscultated indirectly with a stethoscope to detect any deviations from normal in heart sounds or irregularity of rhythm before making the tracings. Each electrocardiogram consisted of tracings from the three standard limb leads. All tracings were made of the animal in a standing position on dry wood shavings in a dairy barn. The dry shavings were employed to prevent 60-cycle electrical interference from appearing on the tracing.

A direct-writing electrocardiograph was employed to make the tracings.* Arrhythmia was recorded as present or absent after examining the tracing from lead II. If lead II was unsatisfactory, then the better of the remaining leads was measured. The longest interval on the lead for any wave, complex, or segment was recorded.

Each electrocardiogram was examined with the assistance of a compass and an electrical magnifier,† which permitted interval measurements to be considered accurate for plus or minus 0.02 sec. The P, P-R, QRS and Q-T intervals were recorded, as well as the appearance and amplitude of the P, Q, R, S, and T waves. The measurements of intervals and amplitudes, and nomenclature of waves used, were in accordance with the recommended procedures of Carter,[5] Burch and Winsor [4] and Marriott.[8]

The electrocardiograms were grouped on the basis of the appearance of the QRS complex in lead II after that suggested by Alfredson and Sykes.[1] The systolic index or the relationship of the Q-T interval to the entire cardiac cycle was evaluated according to the formula devised by Bazett.[2] The atmospheric temperature was recorded at the time the tracings were made each morning.

RESULTS

The heart of each cow was auscultated. As far as could be determined, no abnormal sounds nor irregularities in the rhythms of the hearts could be detected in any of the 45 animals employed as normal individuals. Any deviation in rhythm causing a fluctuation of 0.04 sec. or more in cycle length was arbitrarily considered arrhythmia. In tracings of normal cows very few arrhythmias occurred, appearing in only 17 of the 135 electrocardiograms. The fluctuation in rhythm was not more than 0.10 sec. The normal configuration of the waves of the electrocardiogram was not altered by this slight arrhythmia.

The P-R interval measured in seconds for all normal cows (graph 1) was found to be identical in all three tracings from each of 5 cows. It was quite common to find, in a single cow, variations in P-R intervals for the three tracings of 0.01 to 0.04 sec. One cow revealed a variation in interval length of 0.05 sec. from the shortest to the longest interval of the three tracings. The shortest interval of 0.17 sec. occurred in a 2-year-old cow, and the longest interval, 0.28 sec., appeared in a 12-year-old cow. The range of the P-R intervals for all electrocardiograms

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* Viso-cardiette, Model 51, Electrocardiograph, manufactured by the Sanborn Co., Waltham, Mass.

† Dazor Electric Magnifier, Model M-210-B, manufactured by Dazor Manufacturing Co., St. Louis, Mo.

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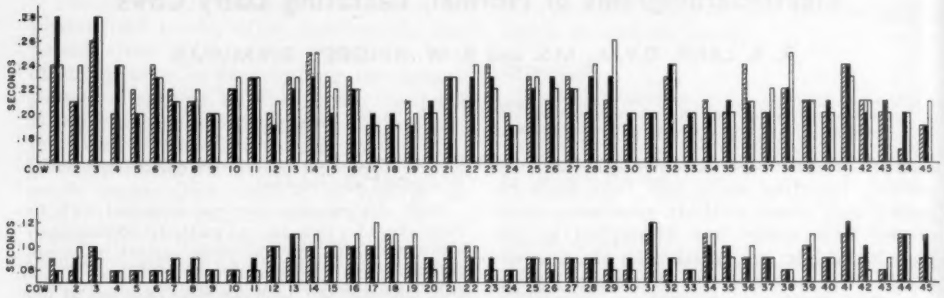


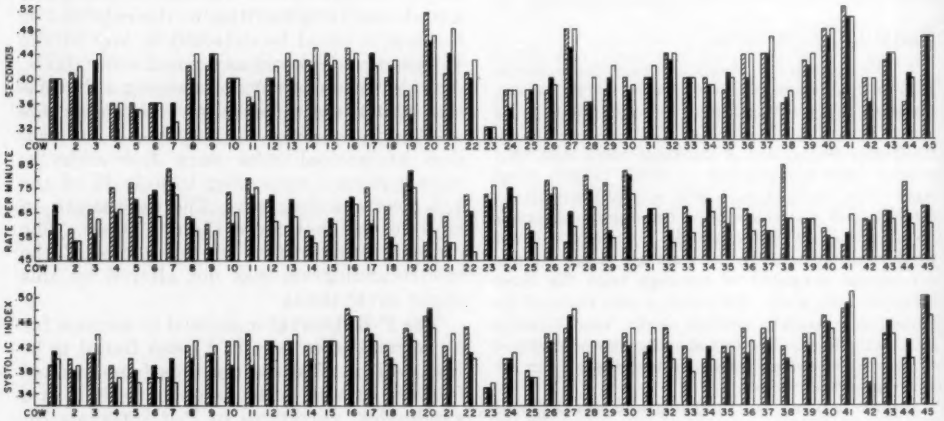
Graph 1—The P-R interval of normal cows. The P-R interval is expressed in seconds for each of the 45 normal cows as measured from lead II of each tracing. The first column above the number of each cow represents the interval on the first tracing; the second column, the interval on the second tracing; and the third column, the interval on the third tracing.

Graph 2—The QRS interval of normal cows. The QRS interval is expressed in seconds for each of the 45 normal cows as measured from lead II of each tracing. The first column above the number of each cow represents the interval on the first tracing; the second column, the interval on the second tracing; and the third column, the interval on the third tracing.

of all normal cows was 0.17 to 0.28 sec. with the average interval being 0.216 sec.

The QRS intervals in seconds for normal cows (graph 2) ranged from 0.08 to 0.12 sec., with an average interval of 0.092 sec. The greatest variation in a single cow on the three tracings was 0.02 sec.

The Q-T interval measurements are recorded graphically in seconds (graph 3). The greatest range of the Q-T intervals in the series of three tracings on any one animal was 0.07 sec. in 2 cows. The same Q-T intervals on all three tracings of an individual animal were seen in 5 cows. A variation



Graph 3—The Q-T interval of normal cows. The Q-T interval is expressed in seconds for each of the 45 normal cows as measured from lead II of each tracing. The first column above the number of each cow represents the interval on the first tracing; the second column, the interval on the second tracing; and the third column, the interval on the third tracing.

Graph 4—The heart rate of normal cows. The heart rate is expressed in cycles per minute for each of the 45 normal cows as determined from lead II of each tracing. The first column above the number of each cow represents the rate on the first tracing; the second column, the rate on the second tracing; and the third column, the rate on the third tracing.

Graph 5—The systolic index of normal cows. The systolic index is expressed as the relationship of the Q-T interval to the cardiac cycle, as computed with Bazett's formula from interval measurements on lead II of each tracing. The first column above the number of each cow represents the index on the first tracing; the second column, the rate on the second tracing; and the third column, the rate on the third tracing.

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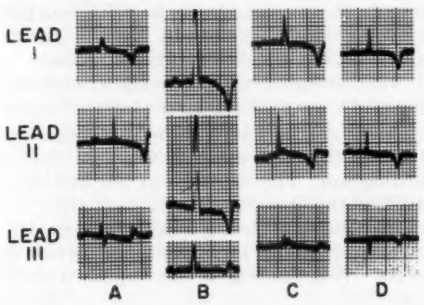


Graph 6—Type 1a normal bovine electrocardiograms. All exhibit a predominant R wave in leads I and II. Lead III has variations in the QRS complex as seen by an RS wave in tracing A, a predominant R wave in tracing B, an M-shaped complex in tracing C, and a predominant Q wave in tracing D.

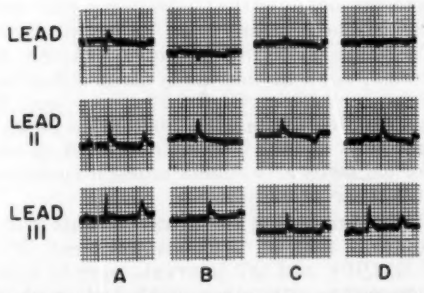
of 0.01 to 0.04 sec. in Q-T interval length was quite common in a single cow. The range of the Q-T intervals on all tracings was 0.32 to 0.52 sec. The average duration of the Q-T interval was 0.409 sec.

The heart rate was recorded as cycles per minute (graph 4). The greatest number of cardiac cycles occurring in any tracings was 84. The slowest rate was 48 cycles per minute. The widest range in any one series of three electrocardiograms was 24. In 1 cow, the rate was the same on all three tracings. The range in heart rates on all electrocardiograms was 48 to 84, the average being 64.5 cycles per minute.

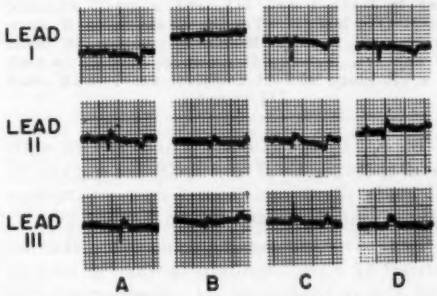
The systolic index on each tracing of all normal cows (graph 5) ranged from 0.35 to 0.51, with the average being 0.42. The greatest range in the index for any 1 animal was 0.05 on the three tracings from 1 cow.

The P wave was usually present in all electrocardiograms; however, in a few leads it was so small in amplitude that it was unmeasurable. The interval of the P wave ranged from 0.06 to 0.12 sec., with an average of 0.082 sec. Changes in the appearance of the wave between cows and, to a lesser degree, between different tracings of the same lead from a single cow were seen frequently.

The QRS complex was invariably present in all leads of the tracings. The electrocardiograms were classified in various types, after the suggestion of previous workers.[1]



Graph 7—Type 1b normal bovine electrocardiograms. All exhibit a predominant R wave in leads II and III. Lead I has variations in the QRS complex as seen by a diphasic wave in tracing A, a Q wave in tracing B, an M-shaped complex in tracing C, and small vibratory deflections in tracing D.



Graph 8—Type 2 normal bovine electrocardiograms. All exhibit a diphasic QRS in lead II. Tracing A has a diphasic QRS complex in lead III and a small RS in lead I. Tracing B has small diphasic QRS in both leads I and III. Tracing C exhibits a large Q wave in lead I and a predominant R wave in lead III. Tracing D shows diphasic QRS in lead I and an M-shaped complex in lead III.

Type 1 showed a predominant R wave in lead II. This category was further divided into groups, type 1a presenting predominant R waves in leads I and II with variations in the QRS complex in lead III (graph 6) and type 1b showing predominant R waves in leads II and III with variations in the QRS complex appearing in lead I (graph 7).

Type 2 was characterized by a diphasic QRS complex in lead II. If the QRS complexes in leads I and III were of the same appearance, either upward or downward, they were usually of small amplitude. A diphasic QRS in lead III was usually accompanied by a small RS in lead I. Other variations recorded were Q wave predominance in lead I and an R in lead III, and a diphasic QRS in lead I with an M shaped complex in lead III (graph 8).

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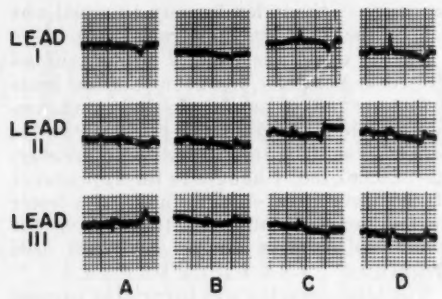
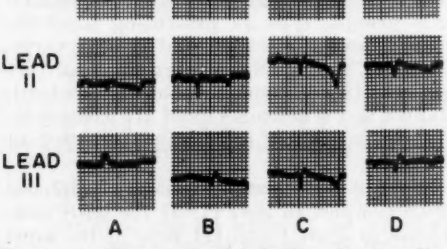


Graph 9—Type 3 normal bovine electrocardiograms. All exhibit small potentials of the QRS complex in all leads. Tracing A has a diphasic QRS in lead I and an M-shaped complex in leads II and III. Tracing B shows an M-shaped complex in leads I and III, and a small diphasic complex in lead II. Tracing C shows an R wave in lead I, and an M-shaped complex in leads II and III. Tracing D shows an R wave in lead I, a vibratory lead II complex, and a RSR' lead III complex.

Type 3 included electrocardiograms with small potentials of the QRS complexes in all leads. Tracings from 4 cows characteristic of this type are shown (graph 9).

Type 4 electrocardiograms were characterized by a predominant Q wave in lead II and also in leads I or III. Tracings of this type are illustrated (graph 10).

The 45 normal cows, grouped as to appearance of the QRS complex in lead II, showed 9 as type 1a, 13 as type 1b, 13 as type 2, 6 as type 3, and 4 as type 4. Variations in the amplitude of the QRS complex occurred from tracing to tracing of the same cow, but the characteristic appearance of the complex did not show sufficient change to warrant a different classification.



Graph 10—Type 4 normal bovine electrocardiograms. All exhibit Q wave predominance in lead II and also in either lead I or lead III. Tracing A shows a Q wave in leads I and II and an M shaped complex in lead III. Tracing B exhibits Q wave predominance in leads I and II, and R wave in lead III. Tracing C shows Q wave predominance in leads II and III, and a small M complex in lead I. Tracing D shows Q wave predominance in all three leads.

The Q and S waves were less frequently observed than any other wave in the electrocardiogram. The S wave did not appear as often as the Q wave. The T wave was present in almost all leads of all tracings, with minor variations in appearance being observed from tracing to tracing of a single cow.

The atmospheric temperatures ranged from 58 to 81 F. at the time the cows were examined and various tracings made.

DISCUSSION

Auscultation of the heart did not reveal any detectable abnormalities, even though a few instances of arrhythmias were shown on the electrocardiograms. Katz and Pick [7] stated that some changes in heart rate due to the influence of the sinoatrial node are, in part, those which should be considered as normal and arise to adjust the activity of the heart to the momentary needs of the animal's body as a partial result of the action of the moderator nerves on the heart. Marriott [8] believed sinus arrhythmias in man to be a perfectly normal finding, with the rate increasing with inspiration and decreasing with expiration. Katz and Pick [7] thought ordinary sinus arrhythmia in man should be considered abnormal if the variation of regularity in the cycle length was more than 0.16 sec. Some normal changes in cycle length, resulting in a degree of arrhythmia in cows, should be expected from both the action of the moderator nerves and the effects of inspiration and expiration. The sinus arrhythmias noticed in the 45 normal cows in this study caused fluctuations in cycle length of 0.10 sec. or less. It is suggested that a cycle fluctuation in normal cows should not be considered as abnormal sinus arrhythmia unless a variation of 0.12 sec. or more occurs.

The duration of the P wave in this study confirms that of previous workers.[9] The P-R, QRS, and QT intervals were in close agreement with other published work.[1,9] Barnes *et al.*[2] found that the P-R intervals lengthened as the animals increased in age. A trend toward longer P-R intervals in older cows was noticed in this study. The QRS interval varied less than any other

interval measured among the series of tracings on any 1 cow. The Q-T interval varied inversely with the heart rate, with a shorter interval generally being associated with a faster heart rate and a longer interval with a slower heart rate. The range of heart rates was found to be in accordance with those published by Dukes.[6]

The systolic index values ranged from 0.35 to 0.51 with an average of 0.42. These values corresponded closely with those previously reported by other workers.[1, 9]

The QRS complex was more variable in appearance that any other segment of the cardiac cycle. The tracings were grouped on the appearances of the QRS complex in lead II, and this study confirms the report of previous workers[1] of the different appearances of the electrocardiogram of the normal dairy animal. It is believed to be a workable classification. The tracings of 77.7 per cent of the 45 animals were of types 1a, 1b, or 2, which confirms the figure of 76.3 per cent for the same groups as previously reported.[1]

Although very little correlation was possible between the appearance of the various waves of the electrocardiograms of cows and those of man, a remarkable similarity in interval length of the two species occurred. The normal interval lengths in electrocardiograms of human beings have been given as a P wave duration of not more than 0.11 sec., a PR interval from 0.12 to 0.20 sec., a QRS complex not exceeding 0.11 sec., and a Q-T interval from 0.35 to 0.45 sec.[8] The intervals of normal lactating dairy cows in this study were similar to those of man.

Summary

The examination of a series of three electrocardiograms on each of 45 normal lactating dairy cows from 2 to 12 years of age, and from nonpregnant to 7 months pregnant cows is reported.

Sinus arrhythmia occurs in normal dairy cows and should not be considered abnormal unless a fluctuation in cycle length of 0.12 sec. or more occurs.

The electrocardiograms resulting from standard limb leads may be grouped quite satisfactorily on the basis of the appearance of the QRS complex in lead II.

The significance of the appearance of the P and T waves in cows was apparently not similar to that in human beings.

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Trial with a New Organic Phosphate as an Anthelmintic in Cattle

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LOSSES DUE TO INFECTION of cattle with species of Trichostrongyloidea have been and continue to be one of the major problems confronting livestock producers. Although programs directed at preventing clinical infections are much more rewarding than treatment of animals after disease occurs, the veterinarian and the owner are often confronted with clinical cases of gastrointestinal nematodiasis which require therapeutic measures.

For nearly two decades, phenothiazine has been the preferred drug for treating infected animals. Although it has been of great value, especially in sheep, a need still exists for a more effective and dependable anthelmintic for use in cattle.

It has been reported [3] that Trolene* would remove Haemonchus, Cooperia, and Ostertagia species from the gastrointestinal tracts of cattle. Other workers [5] have reported on *in vitro* trials against horse strongyle larvae with a variety of organic phosphorus compounds. These trials indicated that certain compounds had a high degree of nematocidal activity.

Herlich and Porter [4] recently reported on critical anthelmintic trials in cattle and sheep treated with thiophosphate, 0,0-diethyl 0-(3-chloro-4-methyl-7-coumarinyl) phosphorothionate (Bayer 21/199).* They found that, in cattle, the drug was highly efficient against *Ostertagia ostertagi* at a dosage of 25.0 mg. per kilogram of body weight but fell off rapidly at dosages of 6.25 and 12.5 mg. per kilogram. In the case of *Cooperia punctata, Cooperia pectinata, Haemonchus placei,* and *Oesophagostomum radiatum,* the material was highly efficient at all dosage levels. Anthelmintic action against *Bunostomum phlebotomum* and *Nematodirus helvetianus* was erratic.

In sheep, it was found that, at a dosage of 25.0 mg. per kilogram, the drug was highly efficient against *Haemonchus contortus, Ostertagia circumcincta, Trichostrongylus axei, Trichostrongylus colubriformis,* and *Cooperia curticei* as well as *Strongyloides papillosus.* At 12.5 mg. per kilogram, the efficiency against *T. colubriformis* and *S. papillosus* was reduced.

The purpose of this paper is to report results of a critical anthelmintic trial in cattle with a new organic phosphate compound.

MATERIALS AND METHODS

The organic phosphate compound used in the trial is still experimental and samples were first sent to federal and state cooperators on July 30, 1956, for tests primarily on cattle grub control. The compound, 0-methyl 0-(4 tert. butyl-2-chlorophenyl) ethylphosphophoramidothioate,† has the following chemical structure:

The $l.d._{50}$ for rats is in the range of 2 Gm. per kilogram.

In this trial, the material was prepared as a 25 per cent wettable powder and was administered by stomach tube at the rate of 120 to 130 mg. per kilogram of body weight (41 Gm. active ingredient per animal).

A total of 60 Hereford heifers which had been on irrigated pasture for several months had an average weight of 688 lb. when delivered to the university. They were purchased for use in this trial and in concurrent trials with phenothiazine, which will be reported in a subsequent paper. Before purchase, random fecal samples showed an average parasitic (Trichostrongyloidea) egg count of 220 eggs per gram (e.p.g.) of feces with a range of less than 50 to 1,650 e.p.g. In addition, an examination of a composite sample revealed the presence of fluke ova *(Fasciola hepatica).* The animals were kept on dry pasture and fed alfalfa hay for a month prior to and during the trial.

Fecal samples were collected from each heifer five times during the nine days preceding treatment. On the basis of the average egg count, five

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* Trolene (Dow-ET-57) is produced by the Dow Chemical Co., Midland, Mich.

* Bayer 21/199 is available from Chemagro Corp., New York, N.Y.

† Dowco 105, produced by Dow Chemical Co., Midland, Mich.

TABLE 1—Average Total Worm Counts and Percentages of Efficiency

Group	No. animals	Av. total worm count (abomasum)	Reduction (%)	Av. total worm count (small intestine)	Reduction (%)	Av. total worm count (abomasum and small intestine)	Reduction (%)
Control	10	9,601	....	776	0	10,377	0
Treated	10	2,896	70	96	88	2,992	71

groups of 10 animals each were set up so that each group had a similar range and average egg count.

Animals in group 1 served as controls for the organic phosphate-treated animals and had an average count of 42.5 e.p.g., with a range of less than 50 to 125 e.p.g. Animals in group 2 were treated with the organic phosphate compound and had an average of 41.8 e.p.g., with a range of less than 50 to 120 e.p.g.

After treatment, the heifers were held for six days and then sent to slaughter. The abomasums and small intestines were collected and returned to the laboratory for examination. Worm counts and species determinations were made from individual animals in a manner similar to that previously described.[1, 2]

Results

The results of the total worm counts in the abomasums and small intestines from animals in groups 1 and 2, as well as the percentage of reduction in worm counts of the treated group are presented (table 1).

Worm counts from the abomasums and small intestines of the control group were 2,970 to 15,870 and 50 to 2,810, respectively; for the treated group they were, correspondingly, 560 to 6,660 and 0 to 280.

Statistical analysis of the difference between means by use of Student's "t" test revealed that the difference in worm counts in both the abomasums and small intestines was highly significant ($P = <0.01$).

Identification of parasite species revealed that *O. ostertagi* was the principal nematode present in the abomasums of animals in both groups, while *C. punctata* and *Cooperia oncophora* were the principal nematodes in the small intestines of control cattle.

The organic phosphate compound may have had a greater anthelmintic efficacy against *T. axei* (the species present in the abomasums) and Cooperia spp. than against *O. ostertagi* (table 2).

Viable adult liver flukes *(F. hepatica)* were found in the bile ducts of heifers from both groups. No evidence of anthelmintic activity against this parasite was detected; however, this does not preclude the possibility of an anthelmintic action against the migrating stages.

At the dosage used, there was definite toxicosis produced in 50 per cent of the animals. On the day following treatment, 1 heifer showed depression, myasthenia, diarrhea, vomiting, and miosis. Atropine sulfate was administered subcutaneously, and recovery occurred after three to four days. Later in the same day, 4 other heifers were observed to be vomiting, bloated, and depressed. Of these animals, 1 was treated for bloat, and the other 3 were left untreated. All 4 had recovered by the following day.

Discussion

It is apparent from the results that the organic phosphate compound used in this trial was highly effective as an anthelmintic against *T. axei, Trichostrongylus capricola, Trichostrongylus vitrinus, C. oncophora, C. punctata* and, to a lesser degree, against *O. ostertagi.*

Unpublished data from trials with phenothiazine which were conducted concurrently revealed an increase in the percentage of immature nematodes present in the

TABLE 2—The Anthelmintic Efficiency of the Organic Phosphate Compound Against Various Species

Item	Av. No. worms in control animals	Population (%)	Av. No. worms in treated animals	Population (%)	Efficiency (%)
Abomasum					
Ostertagia	8,461	88	2,896	100	66
Trichostrongylus	1,140	12	0	0	100
Small intestine					
Cooperia	730	94	94	98	87
Trichostrongylus*	46	6	2	2	96

* Principally *T. capricola* and *T. vitrinus.*

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abomasums, as compared with findings in control animals. The percentage of immature forms found in the organic phosphate-treated animals was similar to that found in the controls. This may indicate that there was a nematocidal effect against the fourth-stage larvae and tissue stages of the parasites involved. If this indirect evidence of action against the immature stages is substantiated by subsequent critical evaluation, it will demonstrate a difference in the anthelmintic action among the organic phosphates, since Herlich and Porter [4] reported that the action against tissue stages of *O. ostertagi* was negligible with the compound used in their experiment.

In general, the results reported in this paper are in close agreement with those reported by Herlich and Porter [4] and support their results on the anthelmintic activity of an organic phosphate against *O. ostertagi, H. placei, T. axei, C. punctata, C. pectinata, Oe. radiatum,* and Trichuris spp. in cattle. Results reported here also add *C. oncophora, T. capricola,* and *T. vitrinus* to the list of nematodes parsitizing the gastrointestinal tracts of cattle which are susceptible to the anthelmintic action of organic phosphate compounds.

Due to the toxicity of the compound tested, further work utilizing lower dosages, and perhaps repeated dosages, are required prior to reaching conclusions in regard to the potential value of this compound under field conditions. However, the present data should serve to adequately substantiate the nematocidal action of this type of organic phosphate compound and stimulate further critical research with these drugs.

Although to the authors' knowledge no cholinesterase activity has been demonstrated in this group of nematodes, the fact that the drug was effective in eliminating them from the gastrointestinal tract would suggest that they either do have such an enzyme or that some analogous system is present. In this respect it is of interest that, although *F. hepatica* has been shown to contain cholinesterase,[6] there was no apparent anthelmintic action against this parasite.

SUMMARY

In a critical anthelmintic trial, a new experimental organic phosphate compound was found to have marked nematocidal activity against several genera of Trichostrongyloidea in cattle. From 90 to 100 per cent of Trichostrongylus spp. in the abomasums and small intestines, as well as 87 per cent of Cooperia spp. in the small intestines and 66 per cent of Ostertagia spp. in the abomasums were removed by a single dose of 120 to 130 mg. per kilogram of body weight.

This dosage produced toxicosis in 5 of 10 animals, but it was severe in only 1 animal.

No action against adult *Fasciola hepatica* was observed.

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Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and Nematodes. I. Eimeria spp. and the Small Intestinal Worm, *Cooperia Punctata*

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CONCURRENT INFECTIONS of host animals with Eimeria and other parasites usually occur under farm conditions. Tunnicliff [11] reported that massive naturally acquired infections with *Cooperia oncophora, Nematodirus helvetianus,* and coccidia produced clinical signs and losses indistinguishable from those of coccidiosis. He was not able to determine whether the worms or the coccidia were the primary cause of the losses and if the large numbers of nematodes would have caused pathological alterations and visible signs of illness in the absence of the coccidiosis.

Riedel [9] found that when chicks were experimentally infected with *Ascaridia galli* at the age of 1 week and, four weeks later, were given oocysts of *Eimeria tenella,* the weight and morbidity records indicated that the nematode-infected chicks were less resistant than the controls. However, when he [10] infected with cecal coccidia first and with *A. galli* six days later, there was no significant difference in resistance to ascarides between coccidia-infected and control birds.

Lotze [8] suggested that, in domestic sheep, the combination of coccidiosis and fly strike may prove fatal to an animal whose coccidial infection alone would not cause death.

Since a knowledge of the intercurrent effects of two kinds of parasites on each other and on the host is of practical importance, two experiments were set up involving Eimeria spp. and *Cooperia punctata,* a nematode inhabiting the small intestine of cattle.

MATERIALS AND METHODS

The 25 calves used in the experiments were grade Jersey or crossbred Jersey-Guernsey males, born at the regional laboratory or purchased from local dairy herds before 3 days of age. They were kept in outdoor, individual, portable pens [3] until the beginning of the experiment or were placed immediately in outdoor concrete-floored pens,[4] with the floors elevated 2 to 3 ft. above the ground. With the exception of 2 calves in experiment 1 that were given Eimeria and were retained in the portable pens, all test calves which were inoculated with Eimeria, Cooperia, or Eimeria and Cooperia combined were kept in the concrete-floored pens. In both experiments, because of lack of such pens, the uninfected control calves were kept in the portable pens throughout the tests. Since it has been observed that calves experimentally infected with coccidia in these pens have a lower death rate than those in the concrete-floored pens,[4] the 2 previously mentioned calves in test 1 that were given Eimeria and were retained in the portable pens were each given the same number of oocysts as the animals in the Eimeria group kept in the concrete-floored pens. This enabled a comparison of infected and uninfected calves in the portable pens and also those of the Eimeria group in the concrete-floored pens.

Eimeria spp. Infection.—The oocysts of Eimeria spp. were obtained from artificially or naturally infected calves at the regional laboratory. These oocysts were sporulated by the method of Hammond and Davis [6] and washed by repeated centrifugation. After excess water was removed, a wooden tongue depressor was used to place the slightly moist material on the calf's tongue. Differential counts of oocysts in the inoculums and in the feces were made by Boughton's modification [2] of Christensen's sugar-flotation method.

In experiment 1, the 7 calves in the three groups that were given Eimeria alone or in combination with Cooperia (table 1) were each given approximately one million oocysts of a culture containing the following species and percentages: *Eimeria bovis,* 30; *Eimeria ellipsoidalis,* 26; *Eimeria auburnensis,* 19; *Eimeria alabamensis,* 17; *Eimeria zurnii* (spherical form), 5; *Eimeria canadensis,* 1; and *Eimeria zurnii* (elliptical form), 1. The inoculations were given in three roughly divided portions on November 27 and 30, and December 1, 1953.

In experiment 2, the number of oocysts was reduced because of the drastic reactions produced by the coccidia in experiment 1. It was determined that the average inoculum in the first experiment consisted of approximately 10,000 oocysts per pound of body weight, so the number was reduced to approximately 3,000 oocysts per pound,

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TABLE 1—A Comparison of Calves Exposed to Eimeria spp., Cooperia Punctata, or a Combination of Both, and Controls

No. of calves in group	Average age (days)	Inoculation	Av. No. fecal samples examined per group	Av. No. of times signs of infection were evident in fecal samples							Average total weekly weight gain or loss*					
							Oocysts				Av. initial wt.					
				Mucus	Blood	Diarrhea	0–100†	100–500	500–1,000	1,000–10,000	10,000+	1	2	3	4	5	6
EXPERIMENT I																	
3	62.0	*Eimeria* spp.**	19.0	1.7	3.7	14.7	7.3	2.7	1.3	7.7	0.0	92.3	9.7	8.0	8.2	.....	22.2	34.2
3	59.0	*Cooperia punctata***	21.3	1.0	2.7	1.3	15.0	4.3	1.3	0.7	0.0	86.7	12.0	13.0	25.0	.....	33.3	43.0
2	57.0	Eimeria-Cooperia**	21.5	3.5	8.0	18.0	5.0	2.5	1.5	11.5	1.0	98.0	9.5	—1.5	0.0	.....	—1.0	7.0
2	58.5	*Eimeria* spp.‡	22.5	1.0	1.5	16.5	9.0	4.5	2.5	5.5	1.0	92.5	7.5	10.0	11.5	.....	23.5	35.5
4	59.8	(Controls‡)	14.0	1.5	0.8	1.3	10.3	1.0	1.3	1.5	0.0	79.8	5.7	10.3	20.2	27.5	31.5	43.7
EXPERIMENT II																		
2	124.5	*Eimeria* spp.**	20.0	0.0	0.0	6.0	10.0	3.5	0.5	5.0	1.0	171.5	2.0	2.0	0.5	—6.0	8.5	.....
2	124.5	*Cooperia punctata***	19.5	0.5	0.0	3.0	16.5	2.5	0.0	0.5	0.0	148.5	7.0	7.5	13.0	1.5	.....	.....
4	131.3	Eimeria-Cooperia**	19.3	0.0	0.0	9.0	9.5	2.8	1.0	5.8	0.3	150.0	—2.7	—4.2	—11.5	—23.0	.....	.....
3	35.1	(Controls‡)	17.7	2.3	2.3	2.7	7.7	5.3	1.7	3.5	0.0	63.0	11.0	14.6	18.3	.....	28.3	.....

* Figures represent pounds gained or lost since initial weighing. † Average oocyst count per loopful. ** In outdoor concrete-floored pens. ‡ In individual portable pens.

or approximately 285,000 to 540,000 per calf. The following species, and percentages of each, were given: *Eimeria alabamensis*, 30; *E. zurnii* (spherical), 19; *E. ellipsoidalis*, 18; *Eimeria cylindrica*, 8; *E. zurnii* (elliptical), 8; *Eimeria subspherica*, 8; *E. bovis*, 7; and *E. auburnensis*, 2. The inoculations were also given in three portions, on February 26 and March 1 and 2, 1954.

Cooperia Punctata Infection.—Cooperia punctata used in these experiments was originally isolated from naturally parasitized cattle. The nematode was then maintained in pure culture by continuous passage through calves raised free of helminths.[5] The number of infective larvae used in the inoculations was determined by dilution count. Larvae were administered to the calves *per os* in water by means of a blowpipe attached to a 15-ml. rubber bulb.

In experiment 1, each calf was given 125,000 *C. punctata* larvae on November 27, 1953; 70,000 on November 30; and 50,000 on December 1 (each ± 500). The average number administered was approximately 2,750 per pound of body weight.

In experiment 2 (table 2), 3 calves were each given 300,000 (± 500) larvae in three inoculations of 150,000, 100,000, and 50,000 larvae. Three other calves were each given 500,000 (± 500) larvae in inoculations of 250,000, 175,000, and 75,000, administered on the same days as the oocysts.

Calf 1340, scheduled to be given only Eimeria, was accidentally given larvae on March 1, and hence was changed to get both larvae and oocysts. Its three inoculations with larvae were given three days later than those to the other calves. It was replaced by calf 1341, which was given coccidia alone on March 1, 4, and 5, 1954, three days later than the others.

During necropsies, fresh tissues for histological sectioning were obtained from sites about every 6 ft. along the intestines and were preserved in Zenker's or Technicon* fixatives. In addition to the examinations of the sections, microscopic examinations were made of fresh scrapings made from the mucosal surface of the digestive tract. Macroscopic observations were made of any pathological conditions resulting from parasitism. The total number of worms present was estimated from the number recovered from aliquots of the contents and washings of the intestines.

RESULTS AND DISCUSSION

The data from both experiments are summarized (table 1). During experiment 1, calf 1331 of the Eimeria group and calf 1337 of the Eimeria-Cooperia group died. The former calf had profuse diarrhea for ten days and passed bloody feces before it died on the twenty fifth day after the inoculation. It had not appeared as weak as another calf (1332) infected with Ei-

* Technicon Fixative is produced by Technicon Chemical Co., Chauncey, N.Y.

meria only, or as weak as calf 1337, and had fewer days when blood, mucus, sloughed tissue, or a combination of these appeared in the feces. Its lungs were normal. Most of the digestive tract was hyperemic. The upper and lower cecum, middle and lower colon, and rectum showed typical gross lesions of coccidiosis. Microscopic examinations of the oocysts in intestinal scrapings revealed that the infection was predominantly *E. bovis*, with *E. canadensis* next in order, and only a few oocysts of *E. ellipsoidalis*. Many merozoites were seen in the region beginning about 8 ft. anterior to the ileocecal valve, with the greatest number occurring in the last 2 ft. of the ileum. Histopathologically, there was extensive coccidial destruction of the mucosa of the ileum, cecum, and colon, with the greatest damage in the lower colon. In this area there was mucosal sloughing and degeneration of the glands, with many oocysts still in the bases of the glands. The cecum showed less damage and fewer oocysts, but the colon showed more damage than similar areas in calf 1337.

Calf 1337 of the Eimeria-Cooperia group passed feces that were extremely diarrheic from postinoculation day 12 until its death on day 31. It passed blood, mucus, and some sloughed tissues during part of this time. It was surprisingly alert until four days before death. At necropsy, the lungs were normal. Many merozoites were found in fresh smears from sites 1 ft. and 24 ft. below the pyloric valve. They were not found 6 ft. below the pyloric valve or in the colon, and were sparse in other sites in the small intestine. Myriads of oocysts of *E. bovis* were located in the cecum and throughout the colon and rectum. Sectioned tissues revealed hyperemia and partial destruction of mucosa in areas of the small intestine, typical damage produced by *E. bovis*.[7] Within 6 inches of the ileocecal valve, there was generalized sloughing of the ileal mucosa. However, the greatest destruction involved the mucosa of the cecum, colon, and rectum, where some areas had sloughed as far down as the muscularis mucosae. The rectum was damaged more than any other area, with edema and leukocytic infiltration being evident. Nearly every microscopic field showed gametogenesis and developing oocysts.

Fig. 1—Sections of a nematode (N), Cooperia punctata, in mucosa of rectum of calf 1337 (Eimeria-Cooperia group), with developing oocysts of Eimeria spp. (E). Magnification scale = 100 μ.

Approximately 720 adult cooperids, about 0.3 per cent of the number of infective larvae administered, were removed from the small intestine; however, large numbers of worms were observed passing with the feces for several days prior to the calf's death. Examination of the sectioned material excised from the rectum showed a coiled worm imbedded in the mucosa (fig.

TABLE 2—The Number of Cooperia Punctata Recovered from Calves Infected with Worms Only and from Calves Infected Simultaneously with Worms and Coccidia (Experiment 2)

Calf No.	No. infective larvae administered	Interval between inoculation and death (days)	Worms recovered		
			No. worms	Percentage of infective larvae administered	Adult (%)
	Cooperia Punctata Group				
1322	500,000	27	183,000	36.6	69.0
1343	300,000	28	151,280	50.4	70.0
	Eimeria-Cooperia Group				
1325	500,000	Survived	.....	.....	.....
1324	500,000	28	232,800	46.6	91.0
1338	300,000	27	154,800	51.6	86.0
1340	300,000	27	144,120	48.0	100.0

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1, 2). Despite this unusual location,[1] it was identified as a cooperid, based on morphology in serial cross sections. It was distinct from a trichurid similarly imbedded in the wall of the distal end of the cecum. Around the cooperid there were numerous developing coccidia (fig. 1, 2), possibly enabling the nematode to penetrate the damaged tissues.

In experiment 2, in order to ascertain the numbers of worms present (table 2), a calf from each of the two groups given Cooperia larvae was killed and examined 27 or 28 days after the initial inoculation. In addition, the youngest calf (1340) in the Eimeria-Cooperia group was killed while moribund, 27 days after its first inoculation.

There was little difference in the number of worms recovered from calves in the two groups; however, of the worms recovered from the 2 Cooperia-infected calves, 30 and 31 per cent were immature, as compared to 0 to 14 per cent immature worms in the 3 calves infected with both classes of parasites.

Nematode infections usually elicit an immune response by the host, producing inhibition of development of a portion of the invading organisms. Perhaps the intercurrent infections with coccidia and nematodes blocked or interfered with such an immune response, thus accounting for the smaller numbers of immature worms.

Neither of the 2 calves given Cooperia showed internal visible damage in the stomach or digestive tract, despite the large numbers of cooperids recovered.

At necropsy, all 3 calves in the Eimeria-Cooperia group showed macroscopic evidence of parasitism, with the oldest calf (1324) having the least damage. In addition to the pathosis to be described later, the small intestines showed scattered, small, yellowish, caseous pustules that were deeply imbedded and usually slightly larger than the macroscopic schizonts of *E. bovis*.[5]

In calf 1340 (Eimeria-Cooperia), the gastrointestinal tract from the abomasum to the lower colon was extremely edematous and, in most areas, had a thick, pale, rubbery appearance. The degree of edema was greater than that previously observed by the authors. When the gastrointestinal tract was opened, there was a sloughing,

Fig. 2—Enlargement of an area in figure 1, showing portion of the upper two sections of the cooperid (N) and an oocyst (arrow) in the cavity enclosing the nematode. The irregular wall of the oocyst was due to fixation. Magnification scale = 20 μ.

croupous membrane in most areas of the upper third of the small intestine, where there were many cooperids. Some were entangled in the membrane.

Most of the nematodes were partly imbedded in the intestinal epithelium, as described by Bailey.[1] The contents of the first 12 ft. of the small intestine were stained with bile. In the small intestine, beginning about 23 ft. from the pyloric sphincter, macroscopic schizonts[5] occurred at about one per linear foot and increased in numbers to the zone 12 ft. above the ileocecal valve, where there were about six per foot.

In calf 1338 (Eimeria-Cooperia), there were more schizonts than in calf 1340. One macroscopic schizont was found only 11 ft. from the pyloric valve. Usually the schizonts are located in the posterior half of the small intestine and, rarely, in the cecum and colon.[5] In this calf the schizonts occurred in increasing numbers, from one to two per low-power (x 15) dissecting microscope field in the zone 12 to 22 ft. from the pylorus to five or six per field in the posterior 20 ft. of the 54-ft. small intestine. Most of the cooperids in the upper small intestine were partially attached, while the ones found in the lower small intestine were not attached to the mucosal surface. Microscopically, there was little destruction of the surface epithelium of the rectum but many oocysts were there, extending as deep as the muscularis mucosae. It is possible that the attack by the coccidia was waning, as the calf was killed late in the cycle of the coccidia.[5]

As judged by intestinal lesions, calf 1324 (Eimeria-Cooperia) had the lightest infection of any animal in this group. Cooperids were found at the proximal end of the small intestine near the pyloric valve. Macroscopic schizonts were scarce and were limited to the last quarter of the small intestine. Merozoites in fresh scrapings were sparse and were found only in the terminal 20 ft. of the small intestine and in the distal end of the cecum.

The differences in average weight gains in the test groups were outstanding in both experiments (table 1). In the first experiment, during the third week after inoculation, it was evident that the calves given Eimeria alone gained about half as much weight as the controls. The weights of those given Cooperia only were not seriously affected, but those given both parasites showed no gains or actual weight losses. This condition continued until the end of the cycles of the protozoa at the end of the experiment. The results in the Eimeria groups in the concrete-floored and portable pens were nearly the same (table 1); however, 1 calf in the concrete pens died as a result of the infection. In the second experiment, differences in average weight gains were noticed as early as the second week, with the Eimeria-Cooperia-infected calves losing weight consistently until the end of the experiment. By the third week, the calves given Eimeria alone had gained only 0.5 lb., animals in the Cooperia group gained 13.0 lb., those given Eimeria and Cooperia lost an average of 11.5 lb., and the control animals gained an average of 18.3 lb. Somewhat similar results continued into the fourth week.

SUMMARY

A total of 25 grade dairy calves was used in two experiments to determine the effects when calves were given Eimeria spp. or *Cooperia punctata* only, or Eimeria and Cooperia combined, as compared to unparasitized controls.

In both experiments, the calves with the mixed infections showed greater clinical signs of parasitism, especially diarrhea and poor or no weight gains, than the calves with single infections. Of the calves with single infections, those with Eimeria spp. exhibited more pathosis than those with Cooperia alone. There were no marked differences in numbers of worms recovered at necropsy; however, in the Cooperia-infected calves, nearly one third of the nematodes were immature, whereas in the Eimeria-Cooperia-infected calves the immature worms ranged between 0 and 14 per cent. This indicated an interference by the Eimeria with the hosts' usual immune response to worm infections.

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Parenteral Benzathine Penicillin V in Bovine Mastitis

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THE TREATMENT of bovine mastitis, too frequently unsatisfactory, has associated difficulties which are familiar to veterinarians engaged in research and a continual source of discouragement to practitioners. Mastitis constitutes an involved problem because of the many obstacles to the accurate determination of cause, to specific and accurate diagnosis or classification, and to therapy. Frequently the disease persists, or apparent recovery may be followed by recurrent infection.

The medical treatment of mastitis with any of the antibacterial agents administered by intramammary infusion has not been completely successful. Bacteriostatic effectiveness of the medicament used and the susceptibility to it of the offending organisms have been complicating factors. The infusion vehicles have not overcome the physical and physiological barriers which prevent complete penetration of the mammary tissue.

Thus, areas of inflammation and of fibrosis, or those in which a remote focus of infection has become established, usually are not reached. Obviously, parenteral treatment with the antibacterial compound that would diffuse from the blood stream into the mammary tissue appears to offer the best approach for exposure of all of the mammary tissues to the medicinal agent.

In early studies, Barker and Dussault[1] treated a cow with a total dose of 2,618,000 units of calcium penicillin in a beeswax and peanut oil base over a period of four days and, on the basis of their results, stated that the lactating bovine mammary gland appeared to be nonpermeable to penicillin. However, Welsh *et al.*,[7] using amorphous and crystalline penicillin G, with total daily doses of 16,250,000 and 17,250,000 units administered subcutaneously in four divided doses to normal cows, reported on the diffusion of penicillin from blood to milk. These authors stated, however, that the parenteral administration of penicillin for the treatment of mastitis was not suggested because "penicillin can be localized in the udder by intramammary infusion without as rapid loss as when the drug is administered parenterally . . ."

Murphy and Stuart[8] suggested the use of penicillin as an agent whose elimination from the body through the milk might be capable of destroying streptococcic udder infection. These authors, using the method of Welsh *et al.*[7] (4 doses daily), were unable to terminate two infections with *Streptococcus agalactiae*, but found that the administration of 42,000,000 units of crystalline procaine penicillin G injected intramuscularly over a period of seven days would accomplish that end. They suggested that the longer-acting forms of penicillin might make possible wider use of this form of administration and that more attention should be given to the possibility and desirability of parenteral or oral treatment of udder infection.

The unsatisfactory facets of intramammary infusion treatments and the advantages of the intramuscular injection of penicillin were stressed by Sadek.[4] Although penicillin concentrations in the milk were demonstrable for only short periods, he found the injection beneficial in the treatment of streptococcic and micrococcic udder infections.

The recent reports of Ullberg *et al.*[5,6] show that intramammary infusions, when pathological changes are present, are dispersed unsatisfactorily; in some cases, inflammatory products and associated debris influenced distribution to a degree that antibiotic treatment by intramammary infusion seemed of dubious value. They theorized that the most effective therapeutic method appears to be the systemic administration of antibiotics. They also postulated that unsuccessful penicillin treatment of mastitis often results from failure of the drug to reach the diseased areas.

Our preliminary studies with normal milking cattle, in which comparison was made between benzathine penicillin V, benzathine penicillin G, and procaine penicillin G,[2] showed that a single intramuscular dose of 3,000,000 units of benzathine penicillin V resulted in significant and fairly uniform penicillactia for 48 hours.

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XUM

TABLE 1—Penicillin Content of Bovine Body Fluids After a Single Intramuscular Dose of 3,000,000 Units of Benzathine Penicillin V

Cow No.	Wt. (lbs.)	Age (yrs.)	Pregnant	Milk (lbs./day)	Pre-Injection	Units of Penicillin per cc. of Blood Serum - Hours - 24	48	72	96	120	144	168
13	975	4	2 mo.	16	0	0.023	0.025	0.023	0	0	0	0
14	1000	6	1 mo.	32	0	0.050	0.074	0.061	0.028	0	0	0
15	1125	5	1 mo.	30	0.041	0.087	0.054	0.037	0	0	0	0
16	950	3	Open	25	0	0.140	0.062	0.038	0	0	0	0
17	1025	3	1 mo.	28	0	0.076	0.058	0.046	0.042	<0.02	0	0
18	1175	7	8 mo.	Drying	0	0.375	0.280	0.136	0.070	0.032	0.054	0.028
Average						0.125	0.093	0.056	0.023	0.008	0.009	0.004

Cow No.		Fibrosis (Degree)	Laboratory Culture	Min. Inhib. Conc. Pen V (Units/ml.)	Highest Conc. Showing Growth (Units/ml.)	Prior to Treatment	Bacteriology (Days) 7	15	21	30	36	Units of Penicillin per cc. of Milk						
13	RF	...	...	.....	.....	...	...	...	...	...	...	RF	0.014	0	0.013	0	0	...	...
	RR	2	HM	0.025	0.0125	HM	HM	HM		HM		RR	<0.01	0	0	0	0	...	...
	LF	...	...	.....	.....	...	HM	HM	...	...	...	LF	0	0.014	0	0	0	...	...
	LR	...	...	.....	.....	...	...	...	...	...	...	LR	<0.01	0	0	0	0	...	...
14	RF	...	HM	.....	.....	HM	HM	HM	HM	HM	HM	RF	0.037	0.031	0.023	0.014	0	...	...
	RR	...	...	.....	.....	...	...	...	...	...	...	RR	0.037	0.030	<0.01	<0.01	0	...	...
	LF	A	HM	0.05	0.025	HM	...	...	...	...	...	LF	0.023	0.028	0.018	<0.01	0	...	...
	LR	...	SNA	0.125	0.0063	SNA	...	...	...	...	...	LR	0.023	0.029	0.020	<0.01	0	...	...
15	RF	...	...	.....	.....	...	...	...	...	...	...	RF	0.024	0.018	<0.01	0	0	...	...
	RR	1	...	.....	.....	...	...	...	...	...	...	RR	0.021	0.015	<0.01	0	0	...	...
	LF	...	HM	0.025	0.0125	HM				HM	HM	LF	0.025	0.020	0	0	0	...	...
	LR	...	...	.....	.....	...	...	...	...	...	...	LR	0.019	0.015	<0.01	0	0	...	...
16	RF	...	...	.....	.....	...	...	...	...	...	...	RF	0.061	0.045	0.013	<0.01	0	...	...
	RR	...	...	.....	.....	...	...	...	...	...	...	RR	0.033	0.027	<0.01	0	0	...	...
	LF	...	...	.....	.....	...	...	...	...	HM	HM	LF	0.060	0.040	0.016	0	0	...	...
	LR	...	M	0.025	0.0125	M	...	...	...	...	...	LR	0.046	0.025	0	0	0	...	...
17	RF	...	...	.....	.....	...	...	...	...	...	...	RF	0.028	0.025	0.019	0.015	0.013	...	...
	RR	...	...	.....	.....	...	...	...	...	...	...	RR	0.023	0.017	0	<0.01	0	...	...
	LF	...	...	.....	.....	...	...	...	...	...	...	LF	0.031	0.023	0.018	0.015	<0.01	...	...
	LR	...	...	.....	.....	...	...	...	...	...	...	LR	0.028	0.016	0.014	<0.01	<0.01	...	...
18	RF	1	...	.....	.....	...	HM	...	HM	...	...	RF	0	0.021	0	0	0	...	...
	RR	1	...	.....	.....	...	HM	...	...	...	...	RR	<0.01	0.016	<0.01	0	0	...	...
	LF	1	...	.....	.....	...	...	HM	...	...	...	LF	<0.01	0.020	<0.01	0	0	...	...
	LR	1	EC	.....	.....	...	...	EC	...	...	...	LR	0	0.023	0	0	0	...	...
												Average		0.024	0.021	0.009	0.004	0.001	...	...

A = atrophy; EC = *Escherichia coli*; M = nonhemolytic micrococci; HM = hemolytic micrococci; and SNA = other types of streptococci.

TABLE 2—Penicillin Content of Bovine Body Fluids After a Single Intramuscular Dose of 6,000,000 Units of Benzathine Penicillin V

Cow No.	Wt. (lbs.)	Age (yrs.)	Pregnant	Milk (lbs./day)	Pre-Injection	Units of Penicillin per cc. of Blood Serum – Hours – 24	48	72	96	120	144	168
7	1325	10	3 mo.	28	0	0.094	0.148	0.098	0.080	0.036	0.027	0
8	1100	8	2 mo.	16	0	0.092	0.110	0.083	0.050	0.030	0.044	0.029
9	1100	9	1 mo.	30	0	0.112	0.109	0.083	0.054	0.024	0.026	0.026
10	1025	4	1 mo.	23	0	0.090	0.107	0.126	0.071	0.031	0	0
11	1000	8	2 mo.	24	0	0.168	0.168	0.108	0.075	0.036	0.028	0.033
12	1100	11	8 mo.	Drying	0	0.174	0.350	0.116	0.064	0.034	0.029	<0.02
Average						0.121	0.165	0.102	0.065	0.031	0.025	0.015

Cow No.	Fibrosis (Degree)	Laboratory Culture	Min. Inhib. Conc. Pen V (Units/ml.)	Highest Conc. Showing Growth (Units/ml.)	Prior to Treatment	Bacteriology (Days) 7	15	21	30	36	Units of Penicillin per cc. of Milk						
7	RF 1	HM	.....	.....	HM	...	...	HM	HM	...	RF	0.021	0.027	0.025	0.016	<0.01	0	...
	RR 4	HM	0.025	0.0125	HM	...	...	...	..	...	RR	0.022	0.030	0.026	0.016	0	0	...
	LF 1	HM	.....	.....	HM	...	...	...	...	HM	LF	0.026	0.032	0.025	0.016	0	0	...
	LR 3	M	0.025	0.0125	M	...	...	...	...	...	LR	0.018	0.027	0.028	0.015	0	0	...
8	RF 2	...	.....	.....	...	...	...	...	...	...	RF	0.023	0.029	0.023	<0.01	<0.01	0	...
	RR 3	HM	0.05	0.025	HM	...	...	...	...	HM	RR	0.019	0.031	0.023	0	0	0	...
	LF 1	...	.....	.....	...	...	...	...	...	...	LF	0.021	0.029	0.019	<0.01	0	0	...
	LR 2	SA	0.0125	0.0063	SA	...	...	...	...	...	LR	0.025	0.024	0.021	<0.01	0.013	0	...
9	RF 1	...	.....	.....	...	...	...	...	SNA	...	RF	0.060	0.043	0.027	0.019	0.013	<0.01	...
	RR 1	...	.....	.....	...	...	SNA	SNA	SNA	SNA	RR	0.049	0.041	0.031	0.020	<0.01	0	...
	LF 1	...	.....	.....	...	...	...	SNA	...	...	LF	0.055	0.046	0.031	0.021	0.013	0.013	...
	LR 1	SNA	4.0	2.0	SNA	HM	...	...	SNA	SNA	LR	0.051	0.050	0.031	0.024	0.015	0.014	...
10	RF ...	...	.....	.....	...	...	...	...	...	...	RF	0.035	0.031	0.038	0.029	0.022	0.014	...
	RR ...	...	.....	.....	...	...	...	...	...	HM	RR	0.035	0.042	0.040	0.027	0.019	0.013	...
	LF ...	...	.....	.....	...	...	...	...	HM	HM	LF	0.034	0.038	0.038	0.024	0.016	0.014	...
	LR 2	HM	0.025	0.0125	HM	...	HM	...	HM	...	LR	0.032	0.045	0.038	0.021	0.017	<0.01	...
11	RF ...	...	.....	.....	...	...	...	...	...	...	RF	0.100	0.047	0.054	0.034	0.021	0.019	...
	RR ...	HM	0.10	0.05	HM	...	...	...	HM	...	RR	0.100	0.035	0.033	0.021	0.019	0.016	...
	LF ...	...	.....	.....	...	...	...	...	...	SNA	LF	0.059	0.039	0.026	0.021	0.015	<0.01	...
	LR ...	...	.....	.....	...	...	...	...	HM	...	LR	0.071	0.035	0.034	0.032	0.018	0.013	...
12	RF ...	HM	0.05	0.025	HM	HM	...	HM	...	HM	RF	0.019	0.016	0.013	0	0	0	...
	RR ...	HM	0.025	0.0125	HM	HM	...	SA	HM	HM	RR	<0.01	0.015	0.014	0	0	0	...
	LF ...	HM	.....	.....	HM	HM	...	HM	HM	HM	LF	0.013	0	0	0	0	0	...
	LR ...	HM	.....	.....	HM	HM	...	HM	HM	...	LR	0.024	0.024	0.014	0	0	0	...
											Average	0.038	0.032	0.027	0.016	0.010	0.006	...

HM = hemolytic micrococci; SA = *Streptococcus agalactiae;* and SNA = other types of streptococci.

TABLE 3—Penicillin Content of Bovine Body Fluids After a Single Intramuscular Dose of 6,000,000 Units of Benzathine Penicillin V

Cow No.	Wt. (lbs.)	Age (yrs.)	Pregnant	Milk (lbs./day)	Pre-Injection	Units of Penicillin per cc. of Blood Serum - Hours - 24	48	72	96	120
11	1000	7	Open	29	0	0.114	0.097	0.088	0.067	0.054
12	975	3	1 mo.	23	0	0.194	0.128	0.114	0.057	0.035
13		9	Open	39	0	0.124	0.086	0.083	0.062	0.04
14		4	2 wks.	22	0	0.192	0.106	0.075	0.031	0
15	1050	4	Open	27	0	0.142	0.115	0.108	0.066	0.056
Average						0.153	0.106	0.094	0.057	0.037

Cow No.		Fibrosis (Degree)	Laboratory Culture	Min. Inhib. Conc. Pen V (Units/ml.)	Highest Conc. Showing Growth (Units/ml.)	Prior to Treatment	Bacteriology (Days) 7	15	21	31		Units of Penicillin per cc. of Milk				
11	RF	...				...	...	...	...	...	RF	0.052	0.028	0.017	0.016	0.011
	RR	...	Not	Not	Not	...	...	...	...	PM	RR	0.039	0.025	0.016	0.016	0.012
	LF	...	Determined	Determined	Determined	...	...	...	...	...	LF	0.025	0.028	0.011	0.011	< 0.01
	LR	...				...	...	...	...	...	LR	0.062	0.038	0.011	0.014	0.011
12	RF	...				PM	...	...	...	...	RF	0.069	0.025	0.022	0	0
	RR	...	Not	Not	Not	...	...	...	...	...	RR	0.071	0.035	0.026	0.011	0
	LF	...	Determined	Determined	Determined	...	...	...	...	...	LF	0.068	0.053	0.029	0.010	0
	LR	...				...	...	...	...	...	LR	0.065	0.035	0.022	0	0
13	RF	...				SNA,PM	SNA	SNA	SNA	SNA	RF	0.050	0.019	0.010	0.014	0
	RR	...	Not	Not	Not	PM	...	...	...	...	RR	0.050	0.021	0.011	0.013	0
	LF	...	Determined	Determined	Determined	...	...	...	...	...	LF	0.059	0.038	0.011	0.013	< 0.01
	LR	...				SNA,PM	SNA	SNA	SNA	SNA	LR	0.048	0.028	< 0.01	0.014	0
14	RF	...	Not	Not	Not	...	...	...	...	...	RF	0.017	0.047	0.017	0	0
	RR	...	Determined	Determined	Determined	...	PM	PM	...	PM	RR	0.014	0.035	0.010	0	0
	LF	...				PM	...	...	PM	...	LF	0.015	0.035	0.011	0.014	0.01
	LR	...				...	...	...	...	...	LR	0.014	0.038	0.010	0	0
15	RF	...				PM	...	...	...	PM	RF	0.110	0.028	0.011	< 0.01	< 0.01
	RR	...	Not	Not	Not	PM	...	PM	PM	PM	RR	0.103	0.035	0.013	0.013	0.011
	LF	...	Determined	Determined	Determined	PM	...	...	...	...	LF	0.084	0.025	0.011	0.011	< 0.01
	LR	A				PM	...	...	...	PM	LR	0.087	0.028	0.015	0.01	0.01
											Average	0.052	0.032	0.015	0.009	0.005

A = atrophy; SA = *Streptococcus agalactiae*; SNA = other types of streptococci; and PM = hemolytic micrococci.

TABLE 4—Penicillin Content of Bovine Body Fluids After Three Intramuscular Doses of 3,000,000 Units of Benzathine Penicillin V

Cow No.	Wt. (lbs.)	Age (yrs.)	Pregnant	Milk (lbs./day)	Pre-Injection	Units of Penicillin per cc. of Blood Serum — Hours — 24	48	72	96	120	144	168
1	1175	6	1 mo.	29	0	0.038	0.080	0.115	0.110	0.075	0.032	0.046
2	1125	8	5 mo.	12	0	0.088	0.156	0.250	0.196	0.168	0.076	0.086
3	1150	6	4 mo.	20	0	0.158	0.265	0.280	0.106	0.042	0.072	0.035
4	1150	9	2 mo.	22	0	0.055	0.083	0.124	0.073	0.062	0.043	0.047
5	1075	7	3 mo.	26	0	0.094	0.112	0.140	0.120	0.076	0.064	0.057
6	1200	10	8 mo.	Drying	0	0.066	0.41	0.40	0.176	0.220	0.22	0.105
Average						0.083	0.184	0.218	0.130	0.107	0.084	0.062

Cow No.		Fibrosis (Degree)	Laboratory Culture	Min. Inhib. Conc. Pen V (Units/ml.)	Highest Conc. Showing Growth (Units/ml.)	Prior to Treatment	Bacteriology (Days) 7	15	21	30	36		Units of Penicillin per cc. of Milk 24	48	72	96	120	144	168
1	RF	...	HM	....	.....	HM	HM	...	...	...	...	RF	0.015	0.033	0.051	0.028	0.022	0.015	<0.01
	RR	...	...	....	.....	...	SNA	SNA	SNA	SNA	SNA	RR	0.013	0.021	0.050	0.032	0.026	0.02	0.013
	LF	...	HM	0.05	0.025	HM	...	...	...	...	...	LF	0.017	0.027	0.045	0.038	0.023	0.015	0.013
	LR	2	...	....	.....	...	...	...	...	...	...	LR	0.015	0.030	0.058	0.028	0.020	0.016	<0.01
2	RF	...	...	....	.....	...	...	...	...	...	...	RF	0.025	0.061	0.044	0.053	0.046	0.022	<0.01
	RR	...	HM	....	.....	HM	...	...	...	...	...	RR	0.018	0.065	0.062	0.035	0.033	0.017	0.018
	LF	...	HM	0.05	0.025	HM	...	...	...	...	...	LF	0.017	0.068	0.072	0.068	0.048	0.024	0.022
	LR	...	HM	....	.....	HM	...	...	...	...	...	LR	0.017	0.074	0.122	0.070	0.043	0.030	0.021
3	RF	...	HM	0.025	0.0125	HM	...	...	...	...	...	RF	0.028	0.074	0.075	0.015	0	0	0
	RR	...	HM	....	.....	HM	...	...	...	...	...	RR	0.022	0.080	0.073	0.014	0	0	0
	LF	...	SNA,HM	0.05	0.025	SNA,HM	SNA	SNA	SNA	SNA	SNA	LF	0.030	0.064	0.080	0.016	0	0	0
	LR	2	...	....	.....	...	...	...	...	...	...	LR	0.028	0.068	0.105	0.021	0	0	0
4	RF	...	SNA,HM	1.6(2.0)-1.6(2.0)	0.8(1.0)-0.8(1.0)	SNA,HM	SNA	SNA	...	SNA	SNA	RF	0.017	0.037	0.048	0.043	0.024	0.017	0.013
	RR	3	HM	0.025	0.0125	HM	...	...	...	...	...	RR	0.016	0.024	0.039	0.045	0.022	0.017	0.015
	LF	...	EC	....	.....	EC	EC	EC	EC	EC	EC	LF	0.015	0.030	0.044	0.047	0.022	0.016	<0.01
	LR	3	HM	....	.....	HM	...	...	...	...	HM	LR	<0.01	0.035	0.049	0.040	0.018	<0.01	0
5	RF	...	HM	....	.....	HM	...	...	...	...	...	RF	0.031	0.045	0.064	0.018	0.022	0.016	<0.01
	RR	1	SNA	2.0	1.0	SNA	...	...	...	...	...	RR	0.031	0.037	0.054	0.018	0.021	0.015	0.013
	LF	...	HM	0.025	0.0125	HM	...	...	...	...	...	LF	0.035	0.045	0.064	0.019	0.026	0.019	0.013
	LR	1	SNA	0.0125	0.0063	SNA	...	...	...	...	...	LR	0.029	0.045	0.056	0.015	0.026	0.017	0.013
6	RF	...	HM	....	.....	HM	HM	HM	SNA	HM	HM	RF	0.015	0.019	0.016	0	0	0	0
	RR	...	HM	....	.....	HM	HM	HM	HM	HM	SNA	RR	0	0.020	0.015	0.02	0	0	0
	LF	...	HM	0.05	0.0125	HM	HM	HM	SNA	HM	HM	LF	0.014	0.019	0.022	0	0	0	0
	LR	...	...	....	.....	...	...	...	...	...	SNA,HM	LR	0	0	0.013	0.015	0	0	0
												Average	0.019	0.043	0.055	0.030	0.018	0.012	0.008

HM = hemolytic micrococci; SNA = other types of streptococci; and EC = *Escherichia coli*.

TABLE 5—Penicillin Content of Bovine Body Fluids After Three Intramuscular Doses of 3,000,000 Units of Benzathine Penicillin V

Cow No.	Wt. (lbs.)	Age (yrs.)	Pregnant	Milk (lbs./day)	Pre-Injection	Units of Penicillin per cc. of Blood Serum — Hours — 24	48	72	96	120	144	168
1	1200	7	7 mo.	19	0	0.053	0.402	0.198	0.136	0.09	0.076	0.048
2	875	3	1 mo.	21	0	0.065	0.11	0.205	0.134	0.126	0.076	0.045
3	1000	3	1 wk.	24	0	0.075	0.062	0.20	0.126	0.115	0.068	0.044
4	1000	6	Open	33	0	0.122	0.112	0.158	0.88	0.10	0.052	0.032
5	990	6	Open	29	0	0.06	0.124	0.205	0.111	0.10	0.13	0.049
6	1100	5	Open	42	0	0.053	0.086	0.107	0.112	0.092	0.066	0.054
7	975	6	Open	29	0	0.07	0.066	0.14	0.093	0.068	0.046	0.04
8	800	3	1 mo.	19	0	0.072	0.126	0.196	0.158	0.124	0.082	0.051
9	950	3	Open	31	0	0.082	0.14	0.156	0.103	0.078	0.069	0.051
10	1100	4	Open	31	0	0.062	0.10	0.12	0.116	0.095	0.065	0.045
Average						0.072	0.113	0.168	0.118	0.099	0.073	0.046

Cow No.	Fibrosis (Degree)	Prior to Treatment	Bacteriology (Days) 7	15	21	31		Units of Penicillin per cc. of Milk					
1	RF ...	PM	...	...	...	...	RF	0.015	0.035	0.024	0.025	0.014	0.015	< 0.01
	RR 2	...	...	...	...	...	RR	0.01	0.035	0.025	0.028	0.018	0.016	0.011
	LF ...	..	...	...	...	...	LF	0.012	0.025	0.044	0.026	0.019	0.016	< 0.01
	LR 2	PM	...	...	...	...	LR	0.014	0.025	0.046	0.026	0.019	0.016	< 0.01
2	RF ...	...	...	...	...	...	RF	0.052	0.098	0.064	0.060	0.026	0.032	0.018
	RR ...	...	...	...	...	...	RR	0.094	0.072	0.061	0.048	0.021	0.029	0.015
	LF ...	...	...	...	...	...	LF	0.033	0.053	0.038	0.063	0.026	0.030	0.018
	LR ...	...	...	...	...	...	LR	0.032	0.043	0.040	0.070	0.027	0.028	0.017
3	RF ...	...	...	...	...	...	RF	0.122	0.043	0.046	0.025	0.015	0.01	< 0.01
	RR 1	...	...	...	...	...	RR	0.089	0.043	0.036	0.021	0.015	0.012	0
	LF ...	...	...	...	...	...	LF	0.115	0.064	0.040	0.025	0.011	0.015	0.010
	LR ...	...	...	...	...	...	LR	0.101	0.053	0.041	0.019	0.012	0.012	< 0.010
4	RF ...	...	...	...	...	...	RF	0.025	0.053	0.019	0.022	0.018	0.014	< 0.01
	RR ...	...	...	...	...	PM	RR	0.035	0.064	0.016	0.017	0.015	0.012	0
	LF A	PM	...	...	PM	PM	LF	0.025	0.048	0.014	0.018	0.015	0.011	0
	LR ...	PM	...	...	...	PM	LR	0.025	0.043	0.015	0.018	0.013	0.010	0
5	RF ...	PM	...	...	...	...	RF	0.031	0.052	0.046	0.025	0.021	0.015	0.011
	RR ...	...	...	...	...	...	RR	0.025	0.071	0.025	0.024	0.021	0.015	0.011
	LF ...	PM	...	...	...	...	LF	0.025	0.047	0.027	0.026	0.021	0.017	0.013
	LR ...	...	...	...	...	...	LR	0.025	0.028	0.022	0.022	0.017	0.015	0.012
6	RF 4	PM	...	PM	PM	PM	RF	0.022	0.025	0.027	0.018	0.014	0.014	< 0.01
	RR ...	...	...	...	...	...	RR	0	0.017	0.022	0.012	< 0.01	0.011	0
	LF ...	...	...	...	...	...	LF	0	< 0.01	0.013	0	0	0	0
	LR ...	...	...	...	...	...	LR	0	0.017	0.020	0.013	< 0.01	0.010	0
7	RF ...	PM	...	...	...	...	RF	0.021	0.035	0.034	0.011	0.010	0	0
	RR ...	...	...	...	...	...	RR	0.019	0.021	0.037	0.012	0.012	< 0.01	0
	LF ...	PM	...	...	...	...	LF	0.014	0.017	0.028	0.013	0.012	0	0
	LR 4	PM	...	...	...	...	LR	0.025	0.043	0.038	0.016	0.016	0.010	< 0.01
8	RF ...	...	...	...	PM	...	RF	0.014	0.038	0.022	0.022	0.022	0.015	0.011
	RR ...	...	...	...	...	...	RR	0.014	0.035	0.021	0.029	0.022	0.015	0.010
	LF ...	...	...	...	...	...	LF	0.012	0.028	0.023	0.026	0.023	0.014	0.010
	LR ...	...	...	...	PM	...	LR	0.01	0.025	0.015	0.017	0.021	0.014	< 0.01
9	RF ...	...	...	...	...	...	RF	0.017	0.053	0.043	0.024	0.015	0.010	0.010
	RR 1	...	...	...	...	...	RR	0.017	0.043	0.036	0.021	0.013	0.010	0
	LF ...	...	...	...	...	...	LF	0.021	0.053	0.046	0.024	0.013	0.011	< 0.01
	LR ...	...	...	...	...	...	LR	0.019	0.048	0.034	0.016	0.011	< 0.01	0
10	RF ...	PM	...	...	...	...	RF	0.019	0.035	0.015	0.014	0.011	0.010	< 0.01
	RR ...	...	...	...	...	...	RR	-	-	-	-	-	-	-
	LF ...	...	...	...	...	...	LF	0.012	0.028	0.015	0.017	0.013	0.014	< 0.01
	LR ...	...	...	...	...	...	LR	0.021	0.035	0.013	0.014	0.011	0.01	0
							Average	0.030	0.040	0.030	0.023	0.016	0.013	0.007

A = atrophy, and PM = hemolytic micrococci. The laboratory culture, minimum inhibiting concentration of penicillin V, and the highest concentration showing growth were not determined.

TABLE 6—Penicillin Content of Bovine Body Fluids After a Single Intramuscular Dose of 3,000,000 Units of Benzathine Penicillin V

Cow No.	Wt. (lbs.)	Age (yrs.)	Pregnant	Milk (lbs./day)	Pre-Injection	Units of Penicillin per cc. of Blood Serum - Hours - 24	48	72
9*	1725	10	6 mo.	12	0	0.04	0.027	0.045
10*	1375	6	3 wks.	50	0	0.144	0.052	0
11*	1625	11	Open	10	0	0.052	0.045	0.066
12*	1550	8	6 mo.	Dry	0	0.068	0.064	0.122
13*	1400	4	Open	8 (Drying)	0	0.11	0.119	0.043
14*	1650	5	6 mo.	Dry	0	0.038	0.064	0.106
15*	1150	4	Open	60	0	0.062	0.043	0.041
16*	1250	7	1 mo.	60	0	0.053	0.031	0.032
Average						0.071	0.056	0.056

Cow No.		Fibrosis (Degree)	Laboratory Culture	Min. Inhib. Conc. Pen V (Units/ml.)	Prior to Treatment	Bacteriology (Days) 7	15	21	31	Units of Penicillin per cc. of Milk			
9*	RF	1	...	...	SA	...	...	...	...	RF	0	0	0
	RR	3	...	...	...	SA	SA	SA	...	RR	0	0	0
	LF	...	...	...	...	W	...	...	...	LF	0	0	0
	LR	3	...	...	SA,F	SA	SA	SA	...	LR	0	0	0
10*	RF	1	...	...	PM	...	PM	...	...	RF	0.029	0.015	0
	RR	3	...	...	PM,W,F	PM	PM	PM	...	RR	0.025	0.015	0
	LF	...	...	...	...	...	PM	...	...	LF	0.020	0.021	0
	LR	2	...	...	...	...	PM	PM	...	LR	0.015	0.015	0
11*	RF	3	...	...	F	...	...	...	...	RF	0.014	0	0
	RR	3	...	...	F	...	...	...	...	RR	0	0	0
	LF	...	...	...	SNA,FL,F	SNA,W	SNA	SNA	...	LF	0	0	0
	LR	3	SA-HEM	0.025-0.05	SA,FL,F	SA,W	SA	SA	...	LR	0.013	0	0
12*	RF	1	...	...	SA	SA	SA,PM	SA,PM	...	RF	0	0	0
	RR	3	...	...	SA	SA	...	SA,F	...	RR	0	0	0
	LF	4	...	...	SA	...	PM	SA,F	...	LF	0	0	0
	LR	3	...	...	SA	SA	SA,PM	SA,F	...	LR	0	0	0
13*	RF	...	...	...	SA	SA	SA	SA	...	RF	0.032	0.021	0.011
	RR	1	SA-HEM	0.05	SA	SA	SA	SA	...	RR	0.028	0.021	0.010
	LF	...	HEM	...	SNA	SA	SA	SA	...	LF	0.031	0.021	0.011
	LR	...	SA-HEM	0.05-0.10	SNA	...	...	...	...	LR	0.026	0.023	0
14*	RF	...	...	...	SNA,PM	SNA	SNA	SNA	...	RF	0	0	0
	RR	...	...	...	...	SNA	...	PM	...	RR	0	0	0
	LF	...	SNA	0.025	...	...	PM	PM	...	LF	0	0	0
	LR	...	SNA	0.006	SNA	SNA	PM	PM	...	LR	0	0	0
15*	RF	...	...	...	SA,FL	SA	SA,PM	SA	...	RF	0.021	0.012	<0.01
	RR	2	...	...	SA	SA	SA	SA	...	RR	0.018	0.011	0
	LF	...	...	...	SA	...	...	...	...	LF	0.025	0.014	<0.01
	LR	2	...	...	SA	SA	SA	SA	...	LR	0.013	<0.01	0.011
16*	RF	...	...	...	SNA,W	SNA,W	SNA	SNA	...	RF	0.018	0.012	0
	RR	2	...	...	...	...	...	PM	...	RR	0.017	0.011	0
	LF	...	...	...	...	...	...	...	...	LF	0.017	0.017	<0.01
	LR	4	...	...	...	...	...	PM	...	LR	0.011	0.012	0
Average											0.012	0.008	0.002

SA = *Streptococcus agalactiae;* SNA = other types of streptococci; PM = hemolytic micrococci; W = watery; FL = flakes; P = pus; and * = cattle showing clinical signs of mastitis. The units of penicillin per cubic centimeter of blood serum and milk were not determined at 96 and 120 hours.

Comparison of the results after the administration of these three penicillin compounds suggested that the development of penicillactia after the administration of benzathine penicillin V might result from active participation of the mammary gland, rather than from a mere threshold mechanism of blood circulatory spill. These results prompted further study with varying dose schedules in cows in different stages of lactation to check these results, to compare penicillin concentrations in milk from fibrotic and from inflamed quarters, and to attempt to determine the effectiveness of benzathine penicillin V in the treatment of mastitis caused by penicillin-susceptible organisms.

MATERIALS AND METHODS

Because environmental circumstances and physical limitations required that small groups of animals be used, the 49 cows involved in this study were handled in seven groups. All were subjected to complete physical examination and special attention was directed to the udder to determine the degree of fibrosis, if any, in each quarter. Immediately before injection, blood samples were drawn to determine whether any antibacterial activity was present, and milk samples were drawn from each quarter for bacteriological culture. Penicillin concentrations of blood serum and of the milk from each quarter were measured at 24-hour intervals; assays were accomplished by the microbiological cup-plate method, using *Sarcina lutea* as the test organism.

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TABLE 7—Penicillin Content of Bovine Body Fluids After Three Intramuscular Doses of 3,000,000 Units of Benzathine Penicillin V

Cow No.	Wt. (lbs.)	Age (yrs.)	Pregnant	Milk (lbs./day)	Pre-Injection	Units of Penicillin per cc. of Blood Serum - Hours - 24	48	72	96	120	144	168
1*	1400	7	Open	65	0.046	0.03	0.023	0.086	0.067	0.055	0.029	0.024
2*	1500	7	2 mo.	30	0.026	0.11	0.192	0.265	0.23	0.126	0.14	0.05
3*	1575	6	Open	60	0.032	0.033	0.052	0.104	0.115	0.32	0.047	0.047
4*	1600	5	5 mo.	4 (Drying)	0	0.037	0.076	0.126	0.128	0.11	0.102	0.06
5*	1650	5	2 mo.	17	0	0.033	0.06	0.094	0.105	0.10	0.078	0.051
6*	1550	10	Open	12	0	0.031	0.055	0.106	0.118	0.08	0.053	0.047
7*	1600	10	1 wk.	26	0	0.11	0.056	0.258	0.128	0.112	0.051	0.29
8*	1775	10	7 mo.	Dry	0	0.09	0.105	0.205	0.196	0.18	0.132	0.20
Average						0.059	0.077	0.155	0.136	0.135	0.078	0.063

Cow No.		Fibrosis (Degree)	Laboratory Culture	Min Inhib. Conc. Pen V (Units/ml.)	Prior to Treatment	Bacteriology (Days) 7	15	21	31		Units of Penicillin per cc. of Milk						
1*	RF	...	...	....	...	FL	...	...	...	RF	0.012	0.011	0.020	0.015	0.01	0.011	0.012
	RR	...	...	....	SA,FL	SA	SA	SA	PM	RR	0.014	0.018	0.013	0.010	0	0	0.010
	LF	...	...	....	SA,W	SA	SA	...	PM	LF	0.021	0.014	0.018	0.011	0	0	0.010
	LR	...	SA,HEM	0.063-0.10	SA,FL	...	...	PM	...	LR	0.023	0.011	0.013	<0.01	0	0.01	<0.01
2*	RF	...	PM	0.05	PM,W	...	PM	...	...	RF	0	0.015	0.020	0.019	0.01	0.012	0
	RR	...	...	....	PM	...	PM	...	SA	RR	0	0.017	0.020	0.016	0	0.011	0
	LF	...	SA,NON-HEM	0.05	SA	...	...	...	...	LF	0	0.021	0.020	0.017	0	0.012	0
	LR	...	SA,HEM	0.05	...	...	...	SNA	...	LR	0	0.021	0.022	0.019	0.01	0.011	<0.01
3*	RF	1	...	....	PM	...	PM	PM	...	RF	0	0.023	0.028	0.018	0.01	0.013	<0.01
	RR	1	...	....	PM	PM	PM	PM	...	RR	0	0.012	0.022	0.013	0	0	0
	LF	...	...	....	PM	...	...	PM	...	LF	0	0.021	0.026	0.017	0.01	0.013	0
	LR	A	PM	0.10	PM	PM	PM	PM	SA	LR	0	<0.01	0.014	<0.01	0	0	0
4*	RF	...	...	....	SA	SA	SA	SA	SA	RF	0	0.021	0.029	0.029	0.021	0.018	0
	RR	...	PM	0.025	PM	...	PM	PM	...	RR	0	0.011	0.022	0.022	0.013	0.021	0.014
	LF	...	...	....	PM	...	...	PM	...	LF	0	0.015	0.027	0.033	0.016	0.025	0.013
	LR	...	...	....	PM	...	PM	PM	...	LR	0	0.023	0.032	0.022	0.017	0.017	0.012
5*	RF	...	...	....	...	...	...	...	...	RF	0	0	<0.01	<0.01	0	0	0
	RR	...	...	....	...	...	SNA,PM	...	...	RR	0	0	0.010	0.010	0	0.015	0
	LF	...	...	....	...	...	PM	...	...	LF	0	0	0.010	<0.01	0	<0.02	0
	LR	3	...	....	PM	...	...	PM	PM	LR	0	0	0.013	0.011	0	0.023	0
6*	RF	2	...	....	PM,P	...	PM	PM	...	RF	0	0	0.017	0.013	0	<0.01	0
	RR	...	...	....	...	...	...	...	...	RR	0	0	0.015	0.014	0	<0.01	0
	LF	...	...	....	...	...	...	...	...	LF	0	0.028	0.024	0.018	0	<0.010	0
	LR	...	...	....	...	...	...	...	...	LR	0	0.023	0.025	0.016	0	<0.01	0
7*	RF	...	...	....	SA	...	PM	...	...	RF	0	0.017	0.027	0.016	0.011	0	0
	RR	2	...	....	...	...	...	...	...	RR	0	0.015	0.033	0.011	0.014	0.010	0
	LF	1	...	....	...	...	...	...	...	LF	0	0.025	0.025	0.013	0.011	<0.01	0
	LR	2	...	....	PM	...	PM	...	...	LR	0	0.019	0.027	0.015	0.013	<0.01	0
8*	RF	...	SA,HEM	0.05	...	...	...	...	...	RF	0	0	<0.01	0	0	0	0
	RR	1	...	....	...	...	...	...	...	RR	0	0	0	0	0	0	0
	LF	...	...	....	...	...	...	...	...	LF	0	0	0	0	0	0	0
	LR	1	SA,HEM	0.05	SA	SA	SA	SA	...	LR	0	0	0	0	0	0	0
Average											0.017	0.012	0.018	0.014	0.005	0.009	0.003

SA = *Streptococcus agalactiae*; SNA = other types of streptococci; PM = hemolytic micrococci; W = watery; F = fibrosis; FL = flakes; P = pus; and * = cattle showing clinical signs of mastitis.

Concomitantly, the organisms isolated from the preinjection milk samples were identified and their *in vitro* susceptibility to penicillin was determined. Milk samples also were drawn from the experimental animals at weekly intervals for four weeks after the initial injection of antibiotic and were cultured bacteriologically.

The complete data on the 49 cows are shown (tables 1-7). All treatment was with benzathine penicillin V in aqueous solution given intramuscularly. The animals showing no clinical signs of mastitis are included in tables 1 to 5. Those reported in table 1 were given a single dose of 3,000,000 units; those in tables 2 and 3, a single dose of 6,000,900 units; and those in tables 4 and 5, three doses of 3,000,000 units at 24-hour intervals. Of the animals showing clinical signs of mastitis, those reported in table 6 were given a single dose of 3,000,000 units and those in table 7 were given three doses of 3,000,000 units at 24-hour intervals.

RESULTS AND DISCUSSION

Because of wide variation among individual cattle, of the differences in clinical pathology, and of the inaccuracies inherent in biological assay methods, definite interpretations and conclusions can not be drawn.*

However, the findings reported in the first three tables support the results of our preliminary study.[2] Following a single intramuscular dose of benzathine penicillin V, the penicillin content of the milk rose and fell more slowly than the level in the blood. When successive doses were given (tables 4, 5), the result was similar but, in some instances, the milk level fell much more slowly than might be expected from the increase in total dose of antibiotic.

In those instances where antibacterial concentrations of penicillin were attained in the milk, as shown by the *in vitro* evaluation of specific bacterial sensitivity, the organisms vanished from subsequent samples. The later demonstration of similar organisms (table 5) may have been due to reinfection, particularly when the time lapse before bacterial return is considered.

The results obtained with the animals showing obvious clinical evidence of mastitis (tables 6, 7) show a marked difference in the penicillin, blood, and milk levels. Here the milk level rose more slowly, attained lower concentrations, and dropped more quickly than in animals without clinical manifestations.

Most significant, we believe, are these observations: (1) In a distinctly atrophied quarter and in late lactation, penicillin continued to appear in the milk (table 1); (2) fibrotic quarters produced milk showing concentrations of penicillin as high or higher than nonfibrotic quarters (tables 2, 4, 5); (3) the udder secretion of dry cows showed no penicillin (tables 6, 7); and (4) inflammatory exacerbations in the udder interfered with the appearance of penicillin in the milk (tables 6, 7), presumably because of the disturbance of secretory function.

In our opinion, these findings lend support to our hypothesis that penicillactia following intramuscular administration of benzathine penicillin V develops not only through a circulatory threshold mechanism but by more direct involvement, probably secretory, of the mammary tissues.

SUMMARY

Findings following the administration of single and multiple intramuscular doses of benzathine penicillin V to 49 cows in seven groups showing udder fibrosis or clinical manifestations of mastitis (1) support the postulate that ensuing penicillactia results from active participation of the mammary gland, rather than from a mere threshold mechanism of blood circulatory spill, and (2) support the studies of other investigators on the effectiveness of penicillin in the eradication of penicillin-susceptible organisms when the required concentrations, as determined by *in vitro* sensitivity of the specific bacteria, are reached in the mammary tissues.

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* For example, the agent showing antibacterial activity in the preinjection blood samples of 4 of the 49 cows was not identified. None of the animals had been subjected to antibacterial therapy of any kind for at least three weeks before the beginning of this study. Control samples run on laboratory techniques showed no results that might serve as cause to question their validity.

SA = *Streptococcus agalactiae*; SNA = other types of streptococci; PM = hemolytic micrococci; W = watery; F = fibrosis; FL = flakes; P = pus; and * = cattle showing clinical signs of mastitis.

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The Screening of Five Drugs for Their Activity Against Bovine Anaplasmosis

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ALTHOUGH EFFECTIVE therapeutic agents for the treatment of bovine anaplasmosis exist in the tetracycline group of antibiotic substances, the expense of these preparations is such that eradication of the carrier state is not always feasible.[1,5] Generally, the economic value of an infected animal limits the amount of broad-spectrum antibiotic administered to that which produces only a suppression of the etiological agent. Upon recovery, an animal which has been given a comparatively low-level dosage retains the causative organism and, consequently, remains a carrier capable of spreading anaplasmosis.[4]

In recognition of the need for additional drugs for the treatment of bovine anaplasmosis, this screening trial was undertaken.

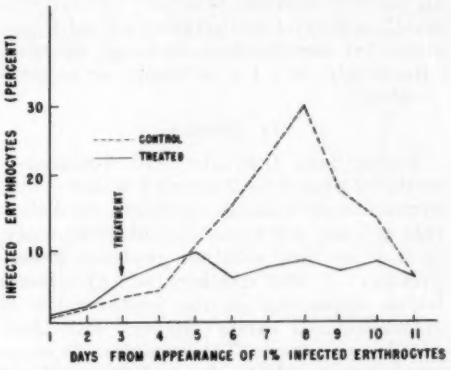
MATERIALS AND METHODS

The procedure followed was that developed by Miller *et al.*[3] In this method, the criterion of effectiveness is based on the limitation of the number of erythrocytes containing Anaplasma bodies when the drug is administered to Anaplasma-infected, splenectomized animals.

Twelve splenectomized calves were infected by intravenous inoculation of citrated whole blood from a calf showing acute anaplasmosis. The inoculum was given at a rate of 10 ml. of infective blood per 100 lb. of body weight. This ratio of the amount of inoculum to the size of the recipient was followed in an effort to produce a more uniform course of the disease in all subject animals.

The drugs were administered at a time when the number of infected erythrocytes reached 5 to 10 per cent, except in 1 animal where the count was 12 per cent at the time of treatment. Two calves were treated with each drug at the manufacturer's recommended dosage, or at an amount well below the estimated toxic level. Two infected, untreated calves served as controls for the entire group.

The course of the disease was followed with daily total erythrocyte counts and packed cell volume, hemoglobin, and percentage of infected erythrocyte determinations. The percentage of infection was obtained by calculating the relative numbers of erythrocytes showing Anaplasma bodies in blood smears, stained with Wright's stain and buffered at pH 6.8. Hemoglobin values were determined photometrically by the acid hematin method.



Graph 1—Comparison of the mean percentages of infected erythrocytes in the control calves and in the calves given 4-amino-1-methyl-2-phenyl-6-(2-amino-1 : 6-dimethyl-pyrimidinium-4-amino)-quinolinium dichloride (Compound 10073).

The preparations tested, with the amount and route of administration for each, were as follows: *p-p'*-diguanyl-diazoamino-benzene (Be 18*) at 3 mg. per kilogram of body weight, intramuscularly, in a 7 per cent solution; acetazoleamide (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide; Diamox†) at 80 mg. per kilogram, intravenously, in a 10 per cent solution; puromycin (Stylomycin†) at 25 mg. per kilogram, intravenously, in a 10 per cent solution; 4-amino-1-methyl-2-phenyl-6-(2-amino-1:6-dimethyl-pyrimidinium-4-amino)-quinolinium dichloride (Compound 10073 **) at 5 mg. per kilogram, subcutaneously, in a 1 per cent solution; and bis-(2-methyl-4-methylaminoquinol-6-yl)-amino-1:1-dimethiodide (Compound 14911**) also at 5 mg. per kilogram, subcutaneously, in a 1 per cent solution.

RESULTS

There was a definite limitation in the number of Anaplasma bodies appearing in

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* Be 18 is produced by E. R. Squibb and Sons, New Brunswick, N.J.; † Diamox and Stylomycin, Lederle Laboratories, Pearl River, N.Y.; ** Compound 10073 and Compound 14911, Imperial Chemical (Pharmaceutical), Ltd., distributed by Fort Dodge Laboratories, Fort Dodge, Iowa.

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the erythrocytes of animals after treatment with 4-amino-1-methyl-2-phenyl-6-(2 amino-1:6-dimethyl-pyrimidinium-4-amino)-quinolinium dichloride at 5 mg. per kilogram, subcutaneously, in a 1 per cent solution (graph 1).

The average shown (table 1) indicates no practical degree of activity for *p-p'*-diguanyl-diazoamino-benzene, puromycin, bis-(2-methyl-4-methylaminoquinol-6-yl)-amino-1:1-dimethiodide (at 5 mg./ kg., subcutaneously, in a 1% solution), or acetazoleamide.

DISCUSSION

These data indicate that 4-amino-1-methyl-2-phenyl-6-(2-amino-1:6-dimethyl-pyrimidinium-4-amino)-quinolinium dichloride at 5 mg. per kilogram, subcutaneously, in a 1 per cent solution produces a suppression of the numbers of Anaplasma bodies appearing in the erythrocytes of splenectomized calves infected with *Anaplasma marginale*. These results are somewhat comparable to those of Brock *et al.*[2] and Miller *et al.*[3] for low-level tetracycline, chlortetracycline, and oxytetracycline. Further studies, using a larger number of animals, should be undertaken to evaluate the drug as to total dosage and frequency of administration required to treat both the acute and carrier states of this disease.

The effect of acetazoleamide is of academic interest. This drug is described as an inhibitor of carbonic anhydrase. It was included in these drug trials on the possibility that, if the Anaplasma body is the specific etiological agent of the disease and is a living entity or group of entities parasitic to the erythrocyte, derangement of the metabolic activity of the cell might prove deleterious to the agent. However, the effect of the disease was intensified by the drug, with death occurring at a time when the total erythrocyte count and hematocrit values may be considered to be critical but not necessarily fatal. Since all other animals in this group survived, including the untreated controls, acetazoleamide appeared to be detrimental to the recovery of the infected calves to which it was given.

Although the three remaining drugs, *p-p'*-diguanyl-diazoamino-benzene, puromycin, and bis-(2-methyl-4-methylaminoquinol-6-yl)-amino-1:1-dimethiodide, showed suppressing activity against Anaplasma in 1 of 2 animals treated with each drug, this effect was canceled out by a lack of action in the second calf. The average (table 1) therefore approximates that of the control animals.

SUMMARY

Five drug preparations were screened

TABLE 1—Hematological Values of Anaplasma-Infected Calves

Drug	Animal (No.)	Highest percentage of Anaplasma bodies	Lowest hematocrit reading	Lowest erythrocyte count	Lowest hemoglobin value
p-p'-diguanyl-diazoamino-benzene					
	442	33.0	9.0	1.27	1.80
	443	14.0	15.0	2.41	4.00
Average		23.5	12.0	1.84	2.90
Acetazoleamide					
	456 *	40.0	8.0	2.25	3.40
	459 *	50.0	9.5	1.85	2.80
Average		45.0	8.8	2.05	3.10
Puromycin					
	433	22.0	9.0	2.00	3.30
	435	49.0	11.0	1.29	2.60
Average		35.5	10.0	1.65	2.95
Compound 10073 †					
	441	16.0	20.0	3.94	6.00
	522	12.0	12.0	1.99	3.00
Average		14.0	16.0	2.97	4.50
Compound 14911 **					
	521	33.0	7.0	1.04	1.00
	431	51.0	9.0	1.22	2.20
Average		42.0	8.0	1.13	1.60
Control group					
	413	24.0	13.0	2.09	3.20
	432	35.0	12.0	1.55	2.80
Average		29.5	12.5	1.82	3.00

* Values immediately preceding death of animal; † 4-amino-1-methyl-2-phenyl-6-(2-amino-1:6-dimethyl-pyrimidinium-4-amino)-quinolinium dichloride; ** bis-(2-methyl-4-methylaminoquinol-6-yl)-amino-1:1 dimethiodide.

for activity in suppressing the numbers of Anaplasma bodies in Anaplasma-infected, splenectomized calves. Of these, 4-amino-1-methyl-2-phenyl-6-(2-amino-1:6-dimethyl-pyrimidinium-4-amino)-quinolinium dichloride (Compound 10073) warrants further study.

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Screening Tests of Carbamates and Related Compounds Against Horse Strongyle Larvae *in Vitro*

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In recent years, a number of carbamic acid esters have been introduced as insecticides.[6] Some, such as Dimetan, Pyrolan, and Isolan, have been used as systemic insecticides in plants,[1,8] but are toxic for warm-blooded animals.[6] However, their insecticidal activity and the fact that the carbamide, diethylcarbamazine, is an effective nematocide, made it appear worthwhile to carry out screening tests of carbamic acid derivatives and related compounds against horse strongyle larvae. The present paper reports the results obtained with 42 of these compounds.

Materials and Methods

The screening technique used in this study has been described by Levine.[2,3] Briefly reviewed, the compound to be tested was mixed with talcum powder to give it body; this mixture was in turn mixed with nine times its weight of fresh horse feces containing strongyle eggs. The resulting mixture was suspended in a cheesecloth bag in a loosely covered, wide-mouthed jar and incubated at 20 C. for a week. At the end of this period, the culture was examined for larvae by the Baermann technique. The cheesecloth bag was placed in a funnel provided with a short length of rubber tubing closed by a pinch clamp. The culture jar was rinsed with warm water, and the rinse water plus additional warm water was poured over the bag to about half its height in the funnel. The preparation was allowed to stand for at least three hours to allow the living larvae to migrate out of the feces and fall down into the column of water in the funnel stem. The liquid from the funnel was collected in a Petri dish and examined under a microscope for larvae. Untreated controls, both with and without talcum powder, were set up and examined with each test. Each compound was tested at concentrations of 0.01, 0.005, 0.0025 and 0.001 molar (M). Lower concentrations were tested later if the compound was found to kill all larvae at these concentrations.

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Dr. Kantor and Dr. Taylor are cooperative agents, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S.D.A. Dr. Kantor is presently at Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y. The present address of Dr. Taylor is Oakland, Ill.

Results

The relation of chemical structure to nematocidal activity of the 25 carbamates studied is shown (table 1). Nine (36%) of these compounds killed all larvae at a concentration of 0.01 M, and four did so at 0.005 M.

Compounds with alkyl substituents on the amino nitrogen were more often active than those with a phenyl group in that position. The only carbamate without any substituent on the amino nitrogen (CBCC 510, 334) was inactive, three of the four compounds with alkyl substituents on the amino nitrogen (G23091, G22008 and Sevin*) were active, but only six of the 20 phenylcarbamates were active.

Among the phenylcarbamates, the addition of chlorine, either to the phenyl group or to the esterifying side chain, did not produce activity. Three compounds of the former type and two of the latter were all inactive.

Insufficient information is available to make it worth while to attempt to formulate any other relationships between chemical structure and nematocidal activity among these carbamates.

The relation of chemical structure to nematocidal activity of the four carbamyl derivatives studied is shown (table 2). Three of them were active. One killed all larvae at 0.01 M, one at 0.005 M and one at 0.0025 M. The only alkyl carbamyl derivative tested was active, as were two of the three phenylcarbamyl derivatives. Since only six of the 20 phenylcarbamates were active, this suggests that carbamyl derivatives may be more active than their related carbamates. However, no direct comparisons were made in the present se-

* Sevin is produced by Union Carbide Chemicals Co.

TABLE 1—Activity of Carbamates Against Horse Strongyle Larvae in Vitro

$$R-\overset{\overset{O}{\|}}{C}-O-R_1$$

Compound No. and source	R	R_1	Lowest lethal concentration	
			Molar	(%)
CBCC* 510,334	NH_2—	$-CH_2CH_2OH$	0.01 inact.	0.10 inact.
UCC† "Sevin"	CH_3NH—	$-C_{10}H_7(\alpha)$	0.005 (0.001 partial)	0.10 (0.02 partial)
CBCC 500,559	$N{=}OCH_2NH$—	$-C_2H_5$	0.01 inact.	0.13 inact.
G** 23091	$(CH_3)_2N$—	2,6-dimethylpyran-(4)-	0.005	0.10
G 22008 (Pyrolan)	$(CH_3)_2N$—	1-phenyl-3-methylpyrazolyl-(5)-	0.005	0.10
CBCC 516,747	C_6H_5NH—	$-CHCH_2{\cdot}C{\equiv}CH$	0.01 inact.	0.19 inact.
CBCC 516,748	m-$CH_3C_6H_4NH$—	$-CH_2C{\equiv}CH$	0.01	0.19
CBCC 910,169	p-ClC_6H_4NH—	$-CH_2C{\equiv}CH$	0.01 inact.	0.21 inact.
CBCC 909,527	m-Cl,o-$CH_3C_6H_3NH$—	$-CH_2C{\equiv}CH$	0.01 inact.	0.22 inact.
CBCC 910,510	m-,o-$(Cl)_2C_6H_3NH$—	$-N{=}C(CH_3)_2$	0.01 inact.	0.26 inact.
L‡ 17754	C_6H_5NH—	$-CH_2CH_2N(CH_3)_2{\cdot}HCl$	0.01 inact.	0.24 inact.
CBCC 500,410	C_6H_5NH—	$-CH_2CH_2OCOCH_3$	0.01 inact.	0.22 inact.
CBCC 910,171	C_6H_5NH—	$-CHCH_2Cl{\cdot}CH_2OCH(CH_3)_2$	0.01 inact.	0.27 inact.
CBCC 909,525	C_6H_5NH—	$-CHCCl_3{\cdot}CH_2NO_2$	0.01 inact.	0.33 inact.
CBCC 516,764	C_6H_5NH—	$-C(CH_3)_2{\cdot}p$-$CH_3C_6H_4$	0.01 inact.	0.27 inact.
L 17129	C_6H_5NH—	$-CHC_6H_5{\cdot}CH_2CH_2N(CH_3)_2{\cdot}HCl$	0.01 inact.	0.33 inact.
L 17393	C_6H_5NH—	$-CHCH_2C_6H_5{\cdot}CH_2N(C_2H_5){\cdot}HCl$	0.01	0.36
L 22972	C_6H_5NH—	$-C{\cdot}CH_3{\cdot}CH_2CH_2C_6H_5{\cdot}CH_2N-(CH_3)_2{\cdot}HCl$	0.01 inact.	0.36 inact.
L 23229	C_6H_5NH—	$-CHC_6H_5{\cdot}CH_2$-piperidino—(N)—·HCl	0.01 inact.	0.36 inact.
L 20206	C_6H_5NH—	$-CHCH_2C_6H_5{\cdot}CH_2$-piperidino—(N)—·HCl	0.01	0.37
L 20307	C_6H_5NH—	$-CHC_6H_5{\cdot}CHCH_3{\cdot}CH_2$-piperidino—(N)—·HCl($\alpha$)	0.01	0.39
L 20274	C_6H_5NH—	$-CHC_6H_5{\cdot}CHCH_3{\cdot}CH_2$-piperidino—(N)—·HCl($\beta$)	0.005	0.20
L 20248	C_6H_5NH—	$-CHCH_2C_6H_5{\cdot}CH_2$-2-methylpiperidino—(N)—·HCl	0.01 inact.	0.39 inact.
CBCC 510,561	$(CH_3)_2CHOCONH$— C_6H_4—m—NH—	$-CH(CH_3)_2$	0.01 inact.	0.28 inact.
CBCC 507,048	$C_6H_5CH_2NH$—	$-CH(CH_3)_2$	0.01	0.19

* CBCC = Chemical-Biological Coordination Center, National Research Council, Washington, D.C.
† UCC = Union Carbide Chemicals Co. (courtesy of Dr. I. H. Roberts, U.S.D.A. ARS Animal Disease and Parasite Research Division, Springfield, Ill.).
** G = Geigy Agricultural Chemicals, New York, N.Y. (Roger Scott).
‡ L = Lilly Research Laboratories, Indianapolis, Ind. (Dr. Max C. McCowen).

ries, and too few compounds were tested to justify more than a tentative conclusion.

The relation of chemical structure to nematocidal activity of the four carbamides studied is shown (table 3). None of these compounds was active against horse strongyle larvae. However, one of them, diethylcarbamazine, was developed as a filaricide and is also effective against ascarides.

Another of these compounds, CBCC 5KO,513, is a potent coccidiostat, being marketed under the name, "Nicarbazin,"* for use in coccidiosis of poultry.

* Nicarbazin is produced by Merck and Co., Inc., Rahway, N. J.

The relation of chemical structure to nematocidal activity of the nine dithiocarbamates studied is shown (table 4). Four of these compounds were active, killing all horse strongyle larvae at a concentration of 0.005 M. The structures of these compounds are too varied to make it possible to draw any solid conclusions on the relationship between chemical structure and nematocidal activity. However, all the active compounds had alkyl substituents on the amino nitrogen. This may have been a factor in their activity.

DISCUSSION

Sixteen (38%) of the 42 compounds

TABLE 2—Activity of Carbamyl Derivatives Against Horse Strongyle Larvae in Vitro

$$R-\overset{\overset{O}{\|}}{C}-R_1$$

Compound No. and source	R	R_1	Lowest lethal concentration	
			Molar	(%)
* L 17167	C_2H_5NH—	$-CHC_6H_5{\cdot}CH_2CH_2$—piperidino—(N)—·HCl	0.005	0.14
L 17351	C_6H_5NH—	$-CHC_6H_5{\cdot}CH_2CH_2$—pyrrolidino—(N)—·HCl	0.01 inact.	0.34 inact.
L 16614	C_6H_5NH—	$-COH{\cdot}C_6H_5{\cdot}CH_2CH_2$—piperidino—(N)—·HCl	0.0025	0.09
L 17271	C_6H_5NH—	$-C(C_6H_5)_2{\cdot}CH_2CH_2$—piperidino—(N)—·HCl	0.01	0.43

* L = Lilly Research Laboratories, Indianapolis, Ind.

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TABLE 3—Activity of Carbamides Against Horse Strongyle Larvae in Vitro

Compound No. and source	R	R_1	Lowest lethal concentration	
			Molar	(%)
* Le 84 (Diethylcarbamazine)	$(C_2H_5)_2N$—	—4-methylpiperazine-(1)-dihydrogen citrate	0.01 inact.	0.24 inact.
† CBCC 506,707	$CH_3 \cdot C_2H_5N$—	—4-methyl-4-oxo-piperazine-(1)	0.01 inact.	0.20 inact.
CBCC 506,710-01	$(C_2H_5)_2N$—	—4-methyl-4-oxo-piperazine-H_2O	0.01 inact.	0.23 inact.
CBCC 5KO,513 (Nicarbazin)	*p*-$NO_2C_6H_4NH$—	—NH-*p*-$NO_2C_6H_4$ (complex with 1 f. wt. 4,6-dimethylpyrimidinol-2)	0.01 inact.	0.30 inact.

* Le = Lederle Laboratories, Pearl River, N.Y. † CBCC = Chemical-Biological Coordination Center, National Research Council, Washington, D.C.

tested were toxic for horse strongyle larvae. This is a much higher percentage than would be found in random testing of miscellaneous compounds and indicates that further study of this group should reveal several practical nematocides.

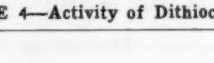
The lowest lethal concentration observed in the present study was 0.0025 M. This is considerably higher than that found by Levine *et al.*[5] for organic phosphorus compounds, in which it was 0.00005 M. However, there is no necessary correlation between extremely high activity in the screening test against horse strongyle larvae *in vitro* and effectiveness *in vivo*. The lowest lethal concentration of phenothiazine in this screening test, for example, is only 0.025 M.[4] The greatest value of the present test is in revealing potentially useful groups of compounds, and it cannot be relied on to pinpoint individual compounds which will be active in the host animal. There are too many differences between the *in vitro* and *in vivo* environments to make this possible.

Several phenylcarbamates have marked local anesthetic action in mammals. Of those which were nematocidal in the present study, L 17393, L 20206, L 20307, and L 20274 were found by Rose *et al.*[7] to produce anesthesia in guinea pig skin for 420, 480+, 420, and 376 minutes, respectively, while of those which were not found to be nematocidal, L 17754, L 17129, and L 20248 produced anesthesia in guinea pig skin for 8, 522, and 455 minutes, respectively. Thus, four of the six phenylcarbamates with mammalian anesthetic properties were also nematocidal, as compared with only one out of the 13 other phenylcarbamates (but of these, the mammalian anesthetic properties of all but one are not known). This correlation may be more than coincidence and may indicate something about the mode of action of these compounds.

SUMMARY

Screening tests for nematocidal activity against horse strongyle larvae *in vitro* were carried out on 42 carbamic acid derivatives and related compounds. Of these, 38 per cent showed some activity at a concentration of 0.01 M or lower. Nine of 25 carbamates, three of four carbamyl derivatives, none of four carbamides and four of 9

TABLE 4—Activity of Dithiocarbamates Against Horse Strongyle Larvae in Vitro



Compound No. and source	R	R_1	Lowest lethal concentration	
			Molar	(%)
* CBCC 803,361-65	CH_3NH—	—Na	0.005	0.065
CBCC 800,352-65	C_4H_9NH—	—Na	0.01 inact.†	0.17 inact.
CBCC 905,273	$(C_2H_5)_2N$—	$-CH_2CH_2OH$	0.005	0.10
CBCC 807,100-65	2-thiozomidazolidinyl-1—CH_2CH_2NH—	$-Na \cdot 3H_2O$	0.01 inact.	0.29 inact.
CBCC 800,087	$(CH_3)_2N$—	$-C(=S)N(CH_3)_2$	0.005	0.10
CBCC 800,036	$(CH_3)_2N$—	$-SC(=S)N(CH_3)_2$	0.005	0.12
CBCC 905,313	$(C_2H_5)_2N$—	$-CH_2CH_2OCH_2CH_2SC(=S)N(C_2H_5)_2$	0.01 inact.	0.37 inact.
CBCC 800,119-74	$(CH_3)_2N$—	$-\frac{1}{2}$ Zn	0.01 inact.	0.15 inact.
CBCC 901,797-60	$(HOCH_2CH_2)_2N$—	$-\frac{1}{2}$ Hg	0.01 inact.	0.28 inact.

* CBCC = Chemical-Biological Coordination Center, National Research Council, Washington, D.C.
† Sample decomposed on receipt.

dithiocarbamates were active. N-alkyl carbamates appeared to be more active than phenylcarbamates. Among the latter, four of six compounds known to produce local anesthesia in the guinea pig were also nematocidal.

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In Vitro Propagation of Hog Cholera Virus. II. Some Biological and Immunological Characteristics of Hog Cholera Virus Grown in Tissue Culture

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THE USE of a relatively simple procedure of the Maitland type for the *in vitro* propagation of hog cholera virus in large volumes, as reported by Dale and Songer,[2] provided a new approach to many new lines of investigations of hog cholera. Among such were studies to determine the most suitable *in vitro* methods for obtaining yields of virus with high infectivity titers, the value of virus so propagated in the production of immunizing agents, and other properties associated with physical, chemical, and biological characteristics of the virus in various types of environment. This paper reports the results obtained in some of the preliminary investigations along these lines.

MATERIALS AND METHODS

As previously described,[2] the virus was cultivated in 250-cc. and 500-cc. Erlenmeyer flasks in a modified Tyrode's medium, with minced pieces of swine spleen as a source of living cells. The total amount of medium used in the 250-cc. flasks was 52.5 cc., which included 2.5 Gm. of spleen and 15.0 cc. of normal swine serum. Four times this amount of medium was used in the 500-cc. flasks. Unless otherwise stated, 250-cc. Erlenmeyer flasks were used for routine passage and 500-cc. Erlenmeyer flasks only where relatively large volumes of virus were needed, such as for the production of hyperimmune serum.

Viruses.—Since several lines of serial passages of viruses from different origins have been made, the procedure of identifying new tissue culture lines with a capital letter, as used previously,[2] has been followed. The number following the capital letter denotes the number of passages in tissue culture mediums.

Procedure for Titration of Virus.—The dilutions were made with buffered saline solution, and 1 cc. of each dilution tested was injected into each of 2 pigs. A titer expressed as 10^{-5} indicates that both pigs injected with this and all lower dilutions died, whereas pigs treated with higher dilutions survived unless otherwise specified. The survivors were subsequently challenged and proved to be susceptible unless otherwise stated.

Special units, similar to those described by Haelterman [4] and which permitted isolation of a single pig, were used for the isolation of some of the pigs. Others were placed in single isolation stalls. Usually 1 of the 2 pigs treated with the same dilution was isolated in a can unit, and the other in an isolation stall.

Occasionally, in a titration test a pig isolated in a unit and injected with the highest dilution that was infective showed varying degrees of illness but recovered, whereas the pig isolated in the stall and injected with the same dilution died of typical acute cholera.

Virus B15.—This virus was established in tissue culture originating from regular hog cholera virus 2266 as described.[2] In the previous studies, another agent was propagated concomitantly with the hog cholera virus through 15 serial passages and the line identified as "B" series.

Virus D.—Rather than isolate a pure culture from the mixture of agents in the B series, another line (D) was established. Like B, this line also originated from regular hog cholera virus 2266.

BAI Regular Virus 304.—BAI regular* virus 304, like virus 2266, was produced at the U.S.D.A. Hog Cholera Research Station, Ames, Iowa.

Experimental Pigs.—All pigs were obtained from the herds at the Agricultural Research Center, Beltsville, Md. They were weaned when 8 weeks old and were from dams immunized with crystal violet-glycerol (CVG) vaccine. In some cases, the pigs used in titration tests were received at the Animal Disease Station, with their dams, when the pigs were 1 to 3 weeks old. When used in titration tests, they had been weaned for not less than three weeks.

Normal Swine Serum.—The serum used as a constituent of the mediums was obtained from cholera-susceptible pigs. After the pigs were exsanguinated, the blood was allowed to stand for several hours. The serum was decanted and, after centrifugation to remove cellular elements, was

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* The term "regular" is applied to hog cholera virus which has not been shown to be an immunological variant. This term was adopted by the authors of an earlier publication [1] after considering terms that might be appropriate. The term "classical" was rejected on the basis that it denoted more specificity than seemed advisable to use at that time.

filtered through a No. 9 Mandler candle and stored at −70 C.

Normal Horse Serum.—The serum was obtained from blood collected from animals maintained at the Animal Disease Station. It was prepared and stored in the same way as the normal swine serum.

Hyperimmune Anti-Hog Cholera Serum.—The hyperimmune antiserums, with the following exceptions, were prepared in general according to prescribed procedures.[6] The pigs were exsanguinated at the first bleeding.

Hyperimmune Serums 3059 and 3063.—These serums were prepared routinely. Three pigs were inoculated with BAI virus 304 to supply the hyperimmunizing virus. Serum 3059 was prepared from 1 pig (318 lb.) by intravenous (i.v.) injection of 5.0 cc. of virus per pound of live weight. Serum 3063 was prepared from 1 pig (253 lb.) by injecting 0.5 cc./lb. i.v. A portion of each serum which was unphenolized was filtered through a No. 8 Mandler candle.

Hyperimmune Tissue Culture Serum 3248.—The hyperimmune tissue culture (TC) serum 3248 was prepared by injecting 2 immune pigs (114 and 119 lb.) with TC virus cultivated from virus B15 (5 cc./lb). The TC virus was cultivated in four 500-cc. Erlenmeyer flasks.[3] This serum was not phenolized.

BAI Experimental Serum 1.—The hyperimmune BAI experimental serum 1 was prepared routinely in a commercial plant in 1949. It has been extensively used to detect hog cholera viruses with variant immunological characteristics.[1,7] As a result, its relative potency is well established. It is used under supervision of federal employees in commercial plants to detect virulent virus with variant characteristics by a test recommended in 1949 by the Pathological Division of the Bureau of Animal Industry. Its use in virus production plants for this purpose was made compulsory in November, 1956.

Methods of Injections.—Viruses and serums were injected into axillary spaces. When given simultaneously, they were injected into opposite areas. The vaccine was injected subcutaneously on the inner surface of the hindleg.

Necropsies.—Necropsies were conducted routinely on each pig at the time of death or at the termination of the experiment. Unless otherwise stated, the tests were terminated at the end of three weeks.

Bacteriological Examinations.—Heart blood, spleen, and lung were routinely cultured on nutrient agar slants.

RESULTS

Potency Tests of Hyperimmune Serums Produced with the Use of Virus Propagated in Tissue Culture.—Test 1.—The objective of test 1 was to determine the potency of a hyperimmune serum produced with the use of virus propagated in tissue culture when used simultaneously with regular hog cholera virus. The results on the 16 pigs (table 1) showed that TC serum 3248, prepared from pigs hyperimmunized with TC virus, possessed sufficient potency to protect 1 of 2 pigs given 15 cc. of serum and both pigs given 30 cc. of serum. Failure to protect 1 of the 2 pigs given 15 cc. suggested that the potency of this serum might not be so great as potencies of serums prepared commercially from regular hog cholera virus.

BAI serum 1 protected 1 of 2 pigs given 5-cc. amounts, both pigs given 15-cc. amounts, and both pigs given 30-cc. amounts.

Serum 3059, prepared by hyperimmunizing a single pig with regular virus, protected both pigs given 15-cc. amounts, as well as both pigs given 30-cc. amounts.

Test 2.—Since the results of the first test failed to provide sufficient evidence to permit a definite conclusion regarding relative potencies of the serum prepared from TC virus and that prepared from regular virus

TABLE 1—Comparative Potencies of Anti-Hog Cholera Serums Prepared with Regular Virus and Tissue Culture Virus (Test 1)

No. pigs	Weight (lb.)	Serum		Virus (2 cc.)	No. pigs giving various reactions				Pigs adequately protected (%)
		Kind	Amount (cc.)		N	Sl	Sv	D	
2	56–75	BAI 1*	5	BAI 304†	1	0	0	1**	50
2	85–90	BAI 1	15	BAI 304	2	0	0	0	100
2	74–80	BAI 1	30	BAI 304	2	0	0	0	100
2	72–99	TC 3248‡	15	BAI 304	0	1	0	1	50
2	66–72	TC 3248	30	BAI 304	2	0	0	0	100
2	76–82	3059§	15	BAI 304	0	2	0	0	100
2	76–77	3059	30	BAI 304	1	1	0	0	100
2	100–105	None	....	BAI 304	0	0	0	2	0

N = normal (no impairment of appetite); Sl = slight reaction; Sv = severe reaction; D = died or killed when moribund (at necropsy all pigs had lesions commonly associated with hog cholera).

* BAI experimental serum 1. † Phenolized virus stored at −40 C. for three years—a dilution of 1:100,000 produced cholera nine months after preparation; a dilution of 1:10,000 produced cholera two months before use in this test. ** The smaller pig (56 lb.) died. ‡ This serum was prepared by pooling the serum from 3 pigs hyperimmunized with hog cholera virus propagated in tissue culture. § This serum was prepared by hyperimmunizing an immune pig with regular virus.

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when used simultaneously with regular virus, a second test was made (table 2). Each of 5 pigs was given 15 cc. of TC serum 3248 and 2 cc. of BAI virus 304. For comparative purposes, 2 pigs were treated with 15 cc. of BAI serum 1, and 2 with 30 cc., and all 4 pigs were given BAI virus 304.

Concurrently with this test, tests were made to determine whether TC virus administered simultaneously with serum would produce lasting immunity in swine, and also to determine the potency of serum from individual immune pigs.

None of the 5 pigs treated with 15 cc. of TC serum 3248 were protected against BAI virus 304; all died between post-treatment days 7 and 12; the 2 control pigs, injected only with virus, died on days 11 and 12. The 2 pigs treated with 15 cc. of BAI serum 1 and BAI virus 304 died on days 11 and 13; the 2 treated with 30 cc. remained normal except for a slight anorexia in 1 pig on one day. In view of the numerous times with opposite results, the failure of 15 cc. of BAI serum 1 to protect the 2 pigs against BAI virus 304 was probably due to their high susceptibility and their heavy weight, rather than to low potency of the serum.

Immunity of Pigs Treated Simultaneously with Tissue Culture Virus and Hyperimmune Serum Prepared from Tissue Culture Viruses.—In a test to determine the immunity of pigs treated simultaneously with TC virus and hyperimmune serum prepared from TC viruses, 14 pigs were used (table 2). Of these, 6 were treated with hyperimmune TC serum 3248 prepared from TC virus; 2 were given 15 cc. each, 2 were given 30 cc. each, and 2 were given 60 cc. each. Six others were treated with BAI serum 1; 2 were given 5 cc. each, 2 were given 15 cc. each, and 2 were given 30 cc. each. These 12 serum-treated pigs were given TC virus D15 simultaneously. Two control pigs were given only TC virus. The 2 pigs treated with 15 cc. of TC serum and TC virus died on post-treatment days 8 and 10; the 4 given larger amounts of serum had slight reactions. The 2 control pigs died on days 9 and 12. Of the 6 pigs treated with BAI serum 1, only the 2 given 5 cc. died (days 10 and 11), while of the 4 pigs given larger amounts of serum, 3 had slight reactions.

When the 8 serum-treated survivors were challenged nine months later with virulent virus, they showed solid immunity; none had a febrile reaction.

Potency of Serum from Immune Pigs.—There is little information on the relative quantities of hog cholera antibodies in serums of pigs immunized by various products and methods. Since this type of information might be helpful in selection of immune pigs that will yield large quantities of antibodies, serums were collected from immune pigs and tested for potency in amounts of 15 cc.

TABLE 2—Comparative Potencies of Anti-Hog Cholera Serums Prepared with Regular Virus and Tissue Culture Virus and of Serums from Nonhyperimmunized Immune Pigs (Test 2)

No. pigs	Weight (lb.)	Serum		Virus (2 cc.)	No. pigs giving various reactions				Pigs adequately protected (%)
		Kind	Amount (cc.)		N	Sl	Sv	D	
5	76–90	TC 3248*	15	BAI 304†	0	0	0	5	0
2**	120–125	BAI 1‡	15	BAI 304	0	0	0	2	0
2**	100–110	BAI 1	30	BAI 304	2	0	0	0	100
2	85–88	None	....	BAI 304	0	0	0	2	0
2	70–70	TC 3248	15	TC D15§	0	0	0	2	0
2	70–74	TC 3248	30	TC D15	0	2	0	0	100
2	72–87	TC 3248	60	TC D15	0	2	0	0	100
2	70–81	BAI 1	5	TC D15	0	0	0	2	0
2	70–75	BAI 1	15	TC D15	0	2	0	0	100
2	70–78	BAI 1	30	TC D15	1	1	0	0	100
2	84–90	None	....	TC D15	0	0	0	2	0
2	89–90	3251	15	BAI 304	0	0	0	2	0
2	83–90	3252	15	BAI 304	0	0	0	2	0
2	71–84	3606	15	BAI 304	0	2	0	0	100
2	70–80	3608	15	BAI 304	0	0	0	2	0

N = normal (no impairment of appetite); Sl = slight reaction; Sv = severe reaction; D = died or killed when moribund (at necropsy all pigs had lesions commonly associated with hog cholera).

* This serum was prepared by pooling the serum from 3 pigs hyperimmunized with hog cholera virus propagated in tissue culture. † Phenolized virus stored at —40 C. for three years—a dilution of 1:100,000 produced cholera nine months after preparation; a dilution of 1:10,000 produced cholera two months before use in this test. ** These pigs, obtained after those in the other groups, were inadvertently provided at heavier weights, which undoubtedly contributed to the failure of the serum to adequately protect the 2 pigs treated with 15-cc. doses. ‡ BAI experimental serum 1. § Fifteenth passage of regular hog cholera virus in tissue culture.

Serums were collected from the following 4 pigs: Pig 3251, vaccinated with 5 cc. of CVG vaccine 101 and challenged three weeks later with BAI virus 304, had a febrile reaction on postchallenge day 4 but was otherwise normal. Pig 3252, vaccinated with 10 cc. of CVG vaccine 101 and challenged three weeks later with BAI virus 304, had a febrile reaction on postchallenge days 3 and 4 but was otherwise normal. The serums of pigs 3251 and 3252 were collected four months after exposure. Pig 3606 was given a vaccine prepared from TC virus D17 and challenged three weeks later with BAI virus 304. It had no reaction after vaccination, but a lengthy febrile reaction after challenge. Pig 3608 was given a vaccine prepared from TC virus D18 and challanged three weeks later with BAI virus 304. It also had no reaction after vaccination, but a severe reaction after challenge. The serum was collected three weeks after exposure.

Two pigs, each treated with 15 cc. of serum from pig 3606, had slight reactions but were adequately protected (table 2). Both pigs had febrile reactions for a duration of eight days and had fair-to-slight appetites for three days. Five of the other 6 pigs died in 11 to 13 days; the sixth pig (1 of the 2 treated with serum 3608) died on day 18 (table 2). It might be of value to know how hogs with these three different types of immunity would respond to hyperimmunization.

Effects of Substitution of Horse Serum for Swine Serum as a Constituent of the Medium.—Since horse serum would have many advantages over the use of swine serum, studies were conducted to obtain some information on this modification.

Virus D10 was used as the inoculum for initiating the line F, where horse serum was used to replace the swine serum. The D line was continued in serial passage as a positive control, and the E line as a negative control (no virus).

Results.—The thirteenth passage of line F produced hog cholera in a single pig injected with 1 cc. Titration of the fifteenth passage showed a titer of 10^{-5}. The thirtieth passage produced hog cholera in a single pig. The course of the disease was typical. The latter pig sickened on the fifth day and died on the tenth day. Necropsy showed lesions commonly associated with hog cholera.

Effects of Refrigeration on Swine Spleen for Use in the Propagation of Hog Cholera Virus in Vitro.—Since there might be occasions when it would be helpful to know whether fresh spleen held in refrigeration for several days could be suitably used in the medium, tests were made to obtain information on this subject.

Procedure.—At the time that routine passages were made with fresh spleen, a portion of the spleen was held under refrigeration. One line (J) was started, using spleen tissue which had been refrigerated at 8 C. for three or four days. A second line (K) was started, using spleen tissue which had been refrigerated at 8 C. for seven days. Virus D14 was used as the original inoculum of both lines. At the same time, line D was used in serial passage as a positive control and line E as a negative control (no virus).

Results.—The seventh serial passage of line J produced hog cholera in a single pig injected with 1 cc. The pig sickened on the sixth day and was killed on the twelfth day, when moribund. Necropsy showed lesions commonly associated with hog cholera. The sixth serial passage of line K failed to produce hog cholera.

Effect of Adding Hyperimmune Serum to Medium.—The classic serum-neutralization test has had no application to hog cholera virus, since the only susceptible animal is the pig. So far, no serum-neutralization test in tissue culture has been developed because of the failure of the virus to produce readily recognizable cytopathogenic effects in types of cells that have been available.

Aside from the possible need for information on a serum-neutralization test of this type, there was need for information on the determination of the comparative differences in amounts of hyperimmune serum required to neutralize regular virus and the variant virus described previously.[1] This information was needed as a preparatory procedure for adding subneutralizing amounts of serum to tissue-culture mediums for serial passage of variant virus in developing a method of replacing the one now used, which consists of treating pigs with virus and subprotective doses of serum.[1]

Procedure.—While the routine serial passages of lines D and E were being made, another flask of medium was seeded with

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2.5 cc. of the D11 inoculum in the same manner as the routine D12 passage was made, but 1.0 cc. of hyperimmune serum 3059 was substituted for 1.0 cc. of the normal swine serum. Three suceeding passages, D12, D13, and D14, were used to inoculate flasks of mediums to which 5.0, 10.0, and 15.0 cc., respectively, of serum 3059 were added, replacing the same amounts of normal serum routinely used in the mediums.

Results.—Titration tests made at a later date showed the D11 and D15 passages to have titers of 10^{-5}. Pig inoculation tests of the serum-virus cultures showed that the virus had been neutralized. This indicated that 1.0 cc. of hyperimmune serum 3059 would neutralize at least 2.5×10^5 m.i.d. In a subsequent test, 0.5 cc. of serum 3059 failed to neutralize the same amount of virus. These preliminary tests provide some information on the potency of hyperimmune serums.

Effects of Prolonged Incubation on Hog Cholera Virus Propagated in Vitro.—It has been accepted by many that the principal factor responsible for the inactivation of the hog cholera virus used in the production of CVG vaccine is the incubation of the product for two weeks at 37 C. Therefore, an attempt was made to determine if, by inactivation at 37 C. for two weeks, the infectivity of the TC virus would be destroyed and its antigenicity maintained.

Procedure.—Aliquots of the culture fluid of serial passages used as inoculum were incubated for varying periods. Some were incubated for one week and others for two weeks.

Results.—Two cubic centimeters of virus D21, incubated for one week, produced hog cholera when injected into a cholera-susceptible pig. Consequently, only virus which had been incubated for two weeks was used as vaccine. The vaccines tested were F7, F8, F9, F10, D17, D18, D19, D20, J1, J2, K1, and E19. Each vaccine was injected in a 10-cc. amount into each of 2 pigs. All pigs remained normal during the three-week postvaccination period. Before challenge with BAI virus 304, none had a febrile reaction. As a result of the challenge, all but 3 pigs died of hog cholera. The 3 pigs that survived were from the groups treated with D17, D18, and D20. Pigs 3606 and 3608, that were vaccinated with D17 and D18, respectively, had febrile reactions which started two or three days after exposure and lasted for several days. One of them was visibly sick for four days. The third pig had a still more severe reaction.

Quantity of Serum Required to Support Propagation of Virus in Tissue Culture.—With a view to determining the minimum amount of serum required in the medium to support optimum virus multiplication, two lines (L and M) of cultures were started.

Procedure.—In these lines, 2.5 cc. of horse serum was used in line L and 5.0 cc. in line M. The F line, in which 15 cc. of horse serum was used, was included as a control. Virus F15 was used as the inoculum of the three lines.

Results.—Sixteen serial passages of L and 15 serial passages of F and M were made. All produced hog cholera when injected into cholera-susceptible pigs. No titrations of infectivity to determine the quantity of virus were made. However, the results showed that the horse serum would support multiplication of hog cholera virus under the procedures used. They also showed that the virus was virulent and pigs injected with the virus succumbed to hog cholera, no attenuation of the virus being observed.

DISCUSSION

The idea of using hog cholera virus propagated *in vitro* in the commercial production of anti-hog cholera serum and better vaccines warranted investigation. Many advantages can be visualized if use of the virus for these purposes could be accomplished. Since the current procedures for the production of the available products in their present forms of immunizing merit were developed after a long period of research into the many phases of improving their efficacy and making their use less hazardous, it is expected that the development of using virus propagated *in vitro* as a substitute in some cases might also require extensive investigations.

The relatively greater difficulties associated with investigations of hog cholera as compared to those in the study of many viral diseases of animals are usually too little realized. One of the greatest obstacles to progress in hog cholera studies is host specificity. Only the pig is susceptible to hog cholera. No laboratory animal serves in detection of the virus, although by adap-

tation, virus multiplication occurs in the rabbit. Added to this refractivity is the absence of any established biological, serological, chemical, or other test, excepting the age-old "cholera-susceptible and cholera-immune" pig test that can be used for the detection of the virus. The resin-coated antibody test [6] and the agar gel diffusion test [5] have not yet been adapted to all experimental needs.

These obstacles are re-emphasized in the early period of initiating *in vitro* propagation of the virus. All *in vitro* passages become blind passages, and the detection of the virus is made known only by pig inoculation, a procedure requiring a minimum period of six to ten days.

Furthermore, results reported herein and in subsequent work [3] show that *in vitro* cytological procedures commonly used successfully with other viruses do not provide a means of virus detection because of failure of the virus to produce a readily recognizable cytodiagnostic effect.

An example of the problems encountered may be found in reviewing the results shown in tables 1 and 2. Failure of the hyperimmune TC serum 3248, prepared with virus propagated *in vitro*, to possess potency comparable to the other serums may be due to one of several causes or a combination of them. A few of the more obvious ones are (1) insufficient virus because of low infectivity titer, (2) the use for hyperimmunization of types of pigs that do not readily lend themselves to heavy yields of antibodies, (3) modification of the virus so that it is relatively less effective in stimulating the production of antibodies, and (4) modification from standard procedure as a result of using a different vehicle for the virus.

Although virus D15, used to inoculate the TC mediums for the production of the hyperimmunizing virus, had a titer of 10^{-5}, as have several other viruses, the titer of the hyperimmunizing virus was not known. It is not known that pigs vaccinated with CVG vaccine and subsequently challenged with virulent virus are a good type of pig to select for hyperimmunization. But in this connection, effort should be made to avoid the selection of pigs like 3606, since its serum was highly potent even without hyperimmunization.

This is another factor that needs to be given proper consideration in this type of study. The relative potency of the serum of the pigs must be known before their hyperimmunization. Otherwise, it may not be possible to make proper evaluation. At this time, it would seem that continued trials will determine whether or not virus propagated *in vitro* will be suitable as hyperimmunizing virus insofar as inherent modification is concerned. The solid immunity of the 4 pigs treated with virus D15 and TC serum suggests that virus propagated *in vitro* may be adequate antigenically as a satisfactory hyperimmunizing agent.

The limited work with the substitution of horse serum for swine serum gave results that are favorable to continuing investigations of this nature. The successful passage of virus in tissue culture, in which the spleen used was refrigerated at least three days but not more than four days, offers the probability that some leeway may be taken in the time of using spleen after collection.

The results obtained from the preliminary tests, in which additions of different quantities of hyperimmune serum were added to the TC medium used for propagation, provide some information for determining brackets for quantitative serum-virus neutralization tests. The results in these tests suggest that 1 cc. of hyperimmune serum will neutralize 2.5×10^5 m.i.d. of virus but not 10×10^5 m.i.d. This, of course, will vary with the potency of the hyperimmune serum.

The survival of a few of the animals vaccinated with vaccines prepared by two weeks' incubation of hog cholera virus propagated *in vitro* may provide a method which may be used to evaluate the antigenicity of virus obtained by various means.

The studies on variations of serum quantity suggest that the amount of serum in the medium required for optimum growth of the virus may be appreciably reduced below the 30 per cent used here for routine propagation.

SUMMARY

Additional studies of *in vitro* propagation of hog cholera virus in a Maitland system of tissue culture are reported. No difficulty was encountered in establishing a new line of serial passage. The eleventh and fifteenth passages of a new line each had infectivity titers of 10^{-5}. The thirtieth passage was highly virulent, and produced

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typical hog cholera and death of affected animals usually within 15 days. In infectivity titration tests of the virus, a decreased pathogenicity was obtained in some cases with the highest dilutions showing infectivity. The course of cholera in some of these pigs was more prolonged and less acute than in pigs given the higher concentration.

By the tests used, hyperimmune serum prepared with virus propagated *in vitro* was somewhat less potent than commercially produced anti-hog cholera serum. Several possible causes for this are suggested, one being the low-infectivity titer of the virus.

No qualitative or quantitative differences in the virus were detected when horse serum was substituted for swine serum in the tissue culture medium. Swine spleen, after refrigeration at 4 C. for three and four days, supported virus propagation, but not after refrigeration for seven days.

Pigs treated simultaneously with virus propagated *in vitro* and anti-hog cholera serum prepared with virus propagated *in vitro* were solidly immune when challenged nine months later.

Vaccines were prepared by incubation of the *in vitro*-propagated virus at 37 C. for two weeks. Of 12 vaccines prepared from 12 different serial passages, three showed immunogenic value, but in each case only 1 of 2 pigs vaccinated survived challenge.

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In Vitro Propagation of Hog Cholera Virus. III. Cultivation of an Immunological Variant, with Retention of Its Identifying Characteristics

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In 1949 and again in 1950, startling losses of swine occurred in areas of the Midwest a few days after vaccination by the use of virulent virus and anti-hog cholera serum, a procedure known as the simultaneous method of vaccination. Investigations indicated that the losses were due to the presence of an immunological variant virus in the simultaneous virus. That hog cholera virus could be used for almost 50 years without discovery of the variant virus caused some doubt [16] of its actual existence, despite the results of extensive tests presented [5] as evidence. However, there still remained need for explanations of many associated problems.

One problem was to determine whether it would be possible to isolate this variant virus (VV) in a form which would maintain itself as a strain, with retention of its particular immunogenic characteristics. Only by passage with a subprotective dose of antiserum was it possible to prevent the virus from reverting to a regular strain of virus.[6] Even when it was passed with antiserum, difficulty was experienced in maintaining the agent so that it would retain its variant immunological characteristics [7] and not revert to regular virus when passed serially from pig to pig. Another characteristic of the variant virus was its consistently low infectivity titer which, perhaps, led to inability to produce with its use a satisfactory crystal violet-glycerol (CVG) vaccine.[8]

Different views were offered as a possible explanation for the instability of the variant virus. One hypothesis suggested that an antigenic component may be unstable like the XII_2 antigen of *Salmonella pullorum*. This might permit the quantity of the antigen to fluctuate, and the degree of fluctuation could be influenced by the quantity of antiserum. It was further suggested that the "variant characteristics," that is, the failure of the virus to be counteracted by a given quantity of serum, an amount previously determined to be capable of counteracting regular virus,* might be enhanced as the quantity of antiserum was gradually increased, thus increasing the quantity of the component of the virus associated with pathogenicity.

A second hypothesis suggested that the variant virus possesses an antigenic component that differs from that of regular virus and hence requires a larger quantity of antiserum to counteract it. It was observed in the field investigations that one serial number of virus was responsible for a 25 per cent loss of the pigs vaccinated, whereas another caused a 5 per cent loss and many others caused losses of less than 1 per cent. To account for a difference in the degree of unfavorable results in herds of swine following the use of the variant virus, it was theorized that a difference would be obtained when the proportions of regular virus and variant virus in a mixture were present in varying amounts. The use of such mixtures, together with the difference in immunological responses in different pigs, would account for the proportions of variation in the numbers of pigs affected.[1]

Regardless of how this quandary may be resolved, it was the first theory, conceived within a month after the first experiment in July, 1949, which was used to design many of the experiments that followed.

The thought that further study of this problem might prove fruitful led to a renewal in 1955 of investigations of the variant virus at Beltsville. Success in the *in vitro* propagation of regular virus [3] offered the possibility of using this procedure in

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* Determined by treatment of pigs with virus and serum injected simultaneously.

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increasing knowledge of the nature of the variant virus and in providing information that might contribute to an understanding of the basic principles involved in the problem of immunological variants. The results obtained in the previous studies [7] suggested the advisability of starting the work with variant 9096, which had proved to be capable of inducing a high percentage of losses when used experimentally.[7,8] The results of additional studies are reported here.

MATERIALS AND METHODS

The serological method of testing viruses for the presence of variant characteristics was the one designed by Dale *et al.*, [5] which consisted merely of injecting pigs simultaneously with varying amounts of a standard hyperimmune serum and with a known regular virus and of injecting similar pigs simultaneously with varying amounts of this serum and the virus being tested. The same serial number of serum, BAI experimental serum 1 (BAI serum 1),[5] used in 1949 as a standard, has been used for this purpose up to the present time (1958). By this method, it was possible to test for immunological differences in viruses by determining the amount of the standard serum required to counteract them. Determining the difference required a large number of pigs, which made each test costly. In order to reduce the cost to a minimum, only a single quantity of the standard serum, 15 cc., was used principally as a screening test. This quantity was selected since most commercial serums, to date (1958), have been tested for potency in this amount; consequently, a mass of information on tests with this amount of serum is a matter of record.

In another publication on the subject of variant virus, Dale *et al.*[6] stated, "If the unknown virus to be tested had variant characteristics, pigs injected with 2 cc. of it simultaneously with 15 cc. of BAI experimental serum No. 1 would not be adequately protected, whereas the contrary would be true if the test material were regular virus." They further stated, "Nevertheless, it is recognized that lesser quantities of serum might show differences in viruses even though they are counteracted by 15-cc. amounts." As investigations of immunological characteristics of viruses are shifted to include viruses propagated in tissue culture, it is important that the facts available as a result of the earlier studies in animals be fully presented in order to permit proper interpretation.

The method used for the propagation of the viruses in tissue culture was the same as that described previously.[3,4] It was a Maitland type system of tissue culture with minced swine spleen as a substrate. Since the variant characteristics (immunological differences) of this virus were maintained only when it was passaged serially in swine simultaneously with a subprotective dose of antiserum, a similar procedure was attempted as part of the *in vitro* propagation studies.

In preliminary *in vitro* tests, it had been determined, using swine as the test animals, that 15.0-, 10.0-, 5.0-, and 1.0-cc. amounts of immune serum 3059 would neutralize 2.5 cc. of a tissue culture (TC) virus having an infectivity titer of 10^{-5}, as indicated by a single animal titration of an early passage. Amounts of 0.5, 0.1, and 0.01 cc. of this serum had failed to neutralize the same quantity of virus. On the basis of these tests, serum in amounts of 1.0, 0.5, 0.2, and 0.1 cc. was added to a series of serial passages of variant virus 3434. Concurrently, a single serial passage of the variant virus without immune serum was made, as well as a single serial passage with immune serum but without virus inoculation. Ten passages of each of these six serial lines were made. The tenth passage of each was inoculated into separate pigs.

Variant Virus 3434.—The origin of this virus is described under "Variant Virus 9096." At the time it was collected, a portion of the defibrinated blood was freed of its cellular elements and the serum stored as inoculating virus of tissue culture mediums. It was not titrated in either form.

Variant Virus 9096.—This virus, described in previous publications,[6,8] was the sixteenth serial passage of variant virus A, a commercial virus. Variant virus A caused large losses among swine when used simultaneously with several different serial numbers of anti-hog cholera serum. In the production of vv 9096, the first nine serial passages of variant virus A were made with subprotective doses of serum.

The immunological variant characteristics of vv 9096 had reverted toward a regular virus as serial passages in swine were continued. Before its use in tissue culture, it was passed serially in swine seven times with 15 cc. of BAI serum 1 and then five times without serum. The last passage, which was identified as vv 3434, made a total of 28 passages of the original variant virus A—the first nine with serum, the next seven without serum, the next seven with serum, and the last five without serum.

The twenty sixth passage was prepared from 4 of 6 pigs injected with the previous passage. Two of these pigs were dead on the sixth day. On the following day, the 4 remaining pigs were exsanguinated. At that time all 4 were off feed, as they had been on the two previous days. A quantity of 2,720 cc. of defibrinated blood, obtained by pooling blood from each pig in proportion to the total amount secured from each animal, was identified as vv 3128. Of this amount, 920 cc. was used as hyperimmunizing virus to prepare hyperimmune serum 3128; 1,200 cc. was used to prepare CVG vaccine 3128; and 600 cc. was distributed in varying amounts in bottles and frozen for virus. An additional quantity of

defibrinated blood from each of the 4 pigs was distributed separately in varying amounts in bottles and frozen for virus, in case the blood of any of these pigs should prove to contain pathogenic bacteria.

Before being pooled, the defibrinated blood of each pig was filtered through two layers of sterile gauze and cultured on agar slants and broth. These cultures yielded no bacterial growth.

Variant Virus 3128.—The description of this virus is given under "Variant Virus 9096." Titration indicated that its titer was at least 10^{-6}.

Variant Virus Hyperimmune Serum 3128.—The vv serum 3128 was prepared from a single pig hyperimmunized with vv 3128 according to the Rules and Regulations Relating to Viruses, Serums, Toxins, and Analogous Products, and to Certain Organisms and Vectors (edition of Nov. 1954), issued by the Animal Inspection and Quarantine Branch, Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C. The pig was exsanguinated at the first bleeding. See additional information on its preparation under the previous heading of "Variant Virus 9096."

BAI Regular Virus 304.—The BAI virus 304, which was obtained from the Hog Cholera Research Station at Ames, was stored at −40 C. or lower, a temperature at which it had been stored since its production. It had an infectivity titer of 10^{-4} at the time of production and also three years later. It was about four years old when used in the present studies.

Regular Virus Hyperimmune Serum 3059.—Serum 3059 had been prepared [4] from a single pig hyperimmunized with a passage of BAI virus 304.

BAI Experimental Serum 1.—The hyperimmune serum (BAI serum 1) was produced for commercial distribution. It was described in a previous publication.[5]

Crystal Violet-Glycerol Vaccine 3128.—The cvg vaccine 3128 was prepared from vv 3128, as described previously.

Experimental Pigs.—All the test pigs were obtained from the herds at the Agricultural Research Center, Beltsville, Md. They were of weaning age, except for a few which were obtained with their dams a few weeks before weaning. All the pigs had been weaned for at least two weeks when used in the tests. Those used in titration tests had been weaned for at least three weeks when inoculated.

Up to the time that hog cholera investigations were discontinued at Beltsville in 1951, no evidence of atrophic rhinitis was observed in the hundreds of pigs that were used. However, *Salmonella choleraesuis* infections, secondary to hog cholera, frequently occurred. Because of the pathogenicity of this microorganism,[15] difficulty was often encountered in interpreting the results of tests in which *S. choleraesuis* was present as a secondary infection.[8] The results of many such tests could not be used for evaluating the hog cholera problem being studied.

Since the renewal of hog cholera investigations in 1955, *S. choleraesuis* has not been encountered. The absence of this infection has been an immense contribution to the current studies, as it has eliminated this factor as an influence on the results. A possible explanation for the disappearance of this bacterial agent from the herd is the replacement of virulent hog cholera virus and serum for immunization against hog cholera by cvg vaccine. Another possible explanation is the introduction of the use of antibiotics in the feed.

During 1951 to 1955, when no hog cholera studies were conducted, atrophic rhinitis became established in the herd. Then, too, ever since the reports in 1948 [12] and 1949 [13] of a chronic pneumonia in swine, there has been ample evidence to suspect a similar disease in the swine herd at Beltsville. With the use of such pigs as experimental animals, it may be justifiable to assume that any manifestation caused by the etiological agents of these entities, in association with hog cholera induced by highly virulent hog cholera virus, are secondary.

Results

Results of Testing Virus Used as Inoculum of Tissue Culture for Deviant Immunological Characteristics.—To ensure the presence of variant immunological characteristics of the virus, it was tested with BAI serum 1.

BAI virus 304 was counteracted by 15 cc. of BAI serum 1, but not by 15 cc. of vv serum 3128; vv 3128 was not counteracted by BAI serum 1 or by serum prepared from this virus in either 15-cc. or 30-cc. amounts. The results indicated that the variant virus was still immunologically different from the regular virus and would be suitable for initiating its propagation in tissue culture.

Test 1.—This test was designed to determine the comparative potencies of a hyperimmune serum prepared from variant virus of swine origin and a hyperimmune serum prepared from regular virus of swine origin when the challenges were variant virus of swine origin and regular virus of swine origin. The weights of the 24 pigs used in the test, the treatments given, and the results are shown (table 1).

The hyperimmune serum prepared from the variant virus failed to protect in amounts of 60 cc. and less against variant virus, even in pigs weighing only 70 lbs. However, this serum in 60-cc. amounts gave complete protection against BAI virus 304 and in 30-cc. amounts protected 1 of 2 pigs. In contrast, the hyperimmune

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serum prepared from regular virus of swine origin gave adequate protection in 30-cc. amounts or less against regular virus of either swine or tissue culture origin.

Test 2.—The objective of this test was to determine whether the immunological differences between a variant virus and a regular virus might be due to quantitative deviations of antigens or other components common to both. To obtain this information, a mixture was made of equal amounts of BAI serum 1, prepared from regular virus, and of vv serum 3128, prepared from variant virus.

The weights of the 32 pigs used in the test, the treatments given, and the results are presented (table 2).

BAI serum 1 in 15-cc. amounts or less gave adequate protection against regular virus but not against variant virus. Amounts of 30 cc. or more gave adequate protection against both viruses. The mixture of serum gave adequate protection against regular virus in 30-cc. amounts, but not in 15-cc. amounts; it gave adequate protection against variant virus in 60-cc. amounts, but not in 30- or 45-cc. amounts. On the basis of the results obtained in this test, vv serum 3128 possessed low potency.

Test 3.—This test with 14 pigs was designed to determine the potency of CVG vaccine prepared from vv 3128 (table 3). When challenged with regular virus, none of the 6 pigs vaccinated with variant CVG vaccine was protected, whereas the 6 treated with the regular CVG vaccine were adequately protected.

The vv serum 3128, prepared with variant virus, was shown to possess low potency (table 2), indicating an inadequacy of certain antigen to induce the production of antibodies. That vv 3128 used in its preparation possessed a high titer supports this view.

Failure of CVG vaccine 3128 to produce any protection in doses of 10 cc. against challenge with BAI virus 304 (table 3) also indicates inadequacy of the virus to elicit antibody response of high potency.

Infectivity Results of Inoculations of Swine with Tenth Passages of Variant Virus and Serum Mixtures.—Of the six lines of serial passages made to study virus propagation in tissue culture containing immune serum, only the tenth serial passage, to which no serum was added, produced hog cholera. The pig in which hog cholera developed was exsanguinated on the seventh day, and the defibrinated blood was identified as vv 4081. Neutralization of the virus, even by the minimum amount of serum, may have occurred because of a low infectivity titer of the virus used for the original tissue culture inoculation. The virus used for this purpose was serum separated from defibrinated blood virus.

TABLE 1—Comparative Potencies of Hyperimmune Serums Prepared with Variant Virus of Swine Origin and with Regular Virus of Swine Origin Against Challenge with Variant Virus of Swine Origin, Regular Virus of Swine Orig'n. and Regular Virus of Tissue Culture Origin

No. pigs	Weight (lb.)	Serum		Virus (2 cc.)	No. pigs giving various reactions				Pigs adequately protected (%)
		Kind	Amount (cc.)		N	Sl	Sv	D	
2	77–87	BAI 1*	15	vv 3128†	0	0	0	2	0
2	77–90	vv serum 3128**	30	vv 3128	0	0	0	2	0
2	70–87	vv serum 3128	60	vv 3128	0	0	0	2	0
2	87–88	None	....	vv 3128	0	0	0	2	0
2	74–84	vv serum 3128	30	BAI 304‡	1	0	0	1	50
2	70–90	vv serum 3128	60	BAI 304	2	0	0	0	100
2	85–88	None	....	BAI 304	0	0	0	2	0
2	70–75	BAI 1	15	TC D15§	0	2	0	0	100
2	70–78	BAI 1	30	TC D15	1	1	0	0	100
2	84–90	None	....	TC D15	0	0	0	2	0
2‖	120–125	BAI 1	15	BAI 304	0	0	0	2	0
2‖	100–110	BAI 1	30	BAI 304	2	0	0	0	100

N = normal (no impairment of appetite); Sl = slight reaction; Sv = severe reaction; D = died or killed when moribund. Pigs were considered adequately protected if they remained normal or had only a slight reaction as a result of the challenge.

* BAI experimental serum 1 is a regular commercial anti-hog cholera serum used as a constant standard. †vv 3128 was the tenth passage of vv 9096, the first seven passages being made with serum. ** vv serum 3128 was prepared with vv 3128. ‡ Phenolized virus stored at —40 C. for three years. A dilution of 1:10,000 produced cholera at the time of preparation and three years later when used in this test. § This was the fifteenth passage in tissue culture of the first passage of BAI virus 304 in swine. ‖ These pigs, obtained after those in the other groups, were inadvertently provided at heavier weights, which undoubtedly contributed to the failure of the serum to adequately protect the 2 pigs treated with 15-cc. doses.

Bacteriology: No bacteriological examinations of the tissues were made.

Since vv 9096 had been passed serially in pigs for an additional seven passages simultaneously with a subprotective dose of hyperimmune serum, it is possible that any residual regular virus had finally been eliminated, leaving only variant virus.

Results of Testing Variant Virus after Serial Passage in Vitro.—A preliminary test to determine whether the deviant immunological characteristics of vv 3128 had been retained after ten passages in tissue culture was made by testing vv 4081 for counteractions by BAI serum 1. BAI serum 1 in doses of 5, 10, or 30 cc. failed to counteract vv 4081, whereas BAI virus 304 was counteracted in 50 per cent of the animals given 5- and 15-cc. amounts and in 100 per cent of the animals given 30-cc. amounts. Death of the 4 pigs treated with 30 cc. of serum and vv 4081, and lack of reactions in the 4 treated with 30 cc. of serum and BAI virus 304, showed that the variant characteristics of vv 3128 had been retained after ten serial passages *in vitro*. The pigs weighed from 68 to 128 lb. Another test was made in which the pigs weighed 87 to 214 lb. In this test, BAI serum 1 in doses of 15, 30, or 60 cc. again failed to counteract vv 4081, whereas BAI virus 304 was counteracted, in 50 per cent of the cases, by 15-cc. amounts and in all cases by 30-cc. amounts.

Test 4.—Since the results of the two preliminary tests failed to indicate the amount of BAI serum 1 required to counteract vv 4081 entirely, a test with 28 pigs was designed to learn if it could be completely counteracted by this serum (table 4).

Neither 15-cc. nor 30-cc. amounts of BAI serum 1 were enough to counteract vv 4081 completely. The 4 pigs treated with 15 cc. of serum and variant virus died of acute hog cholera, and 1 of the 4 treated with 30 cc. had a severe reaction, including a fever that lasted from the third to the twenty fifth day. It acted ill on only two of the first 16 days, but sickened again and was in poor condition when the test was terminated. Two other pigs treated with 30 cc. of serum had febrile reactions from the third to eighth day after treatment; 1 was noticeably sick on four of these days. Febrile reactions for two to six days were

TABLE 2—Comparative Potencies of Hyperimmune Serum Prepared with Variant Virus, of Hyperimmune Serum Prepared with Regular Virus, and of a Mixture of the Two Serums

No. pigs	Weight (lb.)	Serum		Virus (2 cc.)	No. pigs giving various reactions				Pigs adequately protected (%)
		Kind	Amount (cc.)		N	Sl	Sv	D	
2	56–75	BAI 1*	5	BAI 304†	1	0	0	1	50
2	85–90	BAI 1	15	BAI 304	2	0	0	0	100
2	74–80	BAI 1	30	BAI 304	2	0	0	0	100
2	57–81	BAI 1 + vv serum 3128**	5	BAI 304	0	0	0	2	0
2	77–86	BAI 1 + vv serum 3128	15	BAI 304	0	1	0	1‡	50
2	85–85	BAI 1 + vv serum 3128	30	BAI 304	0	2	0	0	100
2	100–105	None	....	BAI 304	0	0	0	2	0
2	60–61	BAI 1	15	vv 3128§	0	0	0	2	0
2	54–60	BAI 1	30	vv 3128	0	2	0	0	100
2	76–77	BAI 1	45	vv 3128	0	2	0	0	100
2	61–62	BAI 1	60	vv 3128	2	0	0	0	100
2	69–71	BAI 1 + vv serum 3128	15	vv 3128	0	0	0	2	0
2	70–71	BAI 1 + vv serum 3128	30	vv 3128	1	0	0	1	50
2	63–66	BAI 1 + vv serum 3128	45	vv 3128	0	0	1	1	0
2	62–63	BAI 1 + vv serum 3128	60	vv 3128	0	2	0	0	100
2	50–53	None	....	vv 3128	0	0	0	2	0

N = normal (no impairment of appetite); Sl = slight reaction; Sv = severe reaction; D = died or killed when moribund. Pigs were considered adequately protected if they remained normal or had only a slight reaction as a result of the challenge.

* BAI experimental serum 1 is a regular commercial anti-hog cholera serum used as a constant standard.† Phenolized virus stored at —40 C. for three years. A dilution of 1:10,000 produced cholera at the time of preparation and three years later when used in this test. ** This serum was a mixture of equal quantities of BAI experimental serum 1 and vv serum 3128. ‡ The pig weighing 86 lb. died. § vv 3128 was the tenth passage of vv 9096, the first seven passages being made with serum.

Bacteriology: Mixed bacterial growth was obtained from pigs that died or were killed when moribund. *Pasteurella multocida* was obtained from 9 pigs, and *Salmonella choleraesuis* from none.

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TABLE 3—Comparative Potencies of Crystal Violet-Glycerol (CVG) Vaccines Prepared with Variant Virus and with Regular Virus Against Challenge with Regular Virus

No. pigs	Vaccines: CVG vaccine 3128 (variant)	Vaccines: CVG vaccine BAI 101 (regular)	Challenge virus: BAI regular virus 304*	No. pigs giving various reactions: N	Sl	Sv	D	Pigs adequately protected (%)
2	10 cc.	....	2 cc.	0	0	0	2	0
2	5 cc.	....	2 cc.	0	0	0	2	0
2	1 cc.	....	2 cc.	0	0	0	2	0
2	..	10 cc.	2 cc.	2	0	0	0	100
2	..	5 cc.	2 cc.	2	0	0	0	100
2†	..	1 cc.	2 cc.	0	1	0	0	100
2	..	....	2 cc.	0	0	0	2	0

N = normal (no impairment of appetite); Sl = slight reaction; Sv = severe reaction; D = died or killed when moribund. Pigs were considered adequately protected if they remained normal or had only a slight reaction as a result of the challenge.

* Phenolized virus stored at —40 C. for three years. A dilution of 1:10,000 produced cholera at the time of preparation and three years later when used in this test. † One pig died before challenge.

Bacteriology: No bacteriological examinations of the tissues were made.

also recorded in 3 of the 4 pigs treated with 60 cc. of serum; in 1 of these pigs the fever persisted, rectal prolapse developed, and the pig died on the thirteenth day, after being visibly sick three days. At necropsy the lesions were not particularly indicative of hog cholera. One of the 4 pigs given 100 cc. of serum had a febrile reaction, but only on the fourth postchallenge day.

Three of the 4 pigs treated with 15 cc. of BAI serum, and 1 given 30 cc., and regular virus had febrile reactions that lasted two to six days. Otherwise, none of these pigs showed evidence of illness.

The 4 virus control pigs died of acute hog cholera. Of the 2 injected with regular virus, 1 died in 10 days and 1 in 11 days; of the 2 given variant virus, 1 died in 11 days and 1 in 13 days.

The results of these tests show conclusively that the immunological variant characteristics of vv 4081 were completely counteracted by the use of a sufficient dose of serum, but that a larger quantity of serum is required to counteract vv 4081 than BAI virus 304.

Necropsy.—Unless otherwise stated, all pigs that died or were killed when moribund had lesions commonly associated with hog cholera.

DISCUSSION

Despite the maintenance of the immunological characteristics of vv 4081, conclusive evidence that it is a stable strain is still lacking. It may be, though most unlikely, that the tissue culture medium used provides an environment which more readily lends itself to the propagation of variant virus than to that of regular virus, which would be opposite to propagation *in*

TABLE 4—Test Showing Variant Virus after Ten Serial Passages in Vitro Completely Counteracted by Increased Amount of Serum Above that Required for Regular Virus

No. pigs	Weight (lb.)	Serum: Kind	Serum: Amount (cc.)	Virus (2 cc.)	No. pigs giving various reactions: N	Sl	Sv	D	Pigs adequately protected (%)
4	75–90	BAI 1*	15	BAI 304†	4	0	0	0	100
4	85–90	BAI 1	30	BAI 304	4	0	0	0	100
2	75–90	None	....	BAI 304	0	0	0	2	0
4	80–80	BAI 1	15	vv 4081**	0	0	0	4	0
4	82–90	BAI 1	30	vv 4081	3	0	1	0	75
4‡	90–95	BAI 1	60	vv 4081	3	0	0	0	100
4	76–90	BAI 1	100§	vv 4081	4	0	0	0	100
2	70–85	None	....	vv 4081	0	0	0	2	0

N = normal (no impairment of appetite); Sl = slight reaction; Sv = severe reaction; D = died or killed when moribund. Pigs were considered adequately protected if they remained normal or had only a slight reaction as a result of the challenge.

* BAI experimental serum 1 is a regular commercial anti-hog cholera serum used as a constant standard.† Phenolized virus stored at —40 C. for three years. A dilution of 1:10,000 produced cholera at the time of preparation and three years later when used in this test. ** This virus was obtained as defibrinated blood from pig 4081, which was injected with the tenth serial passage of variant virus *in vitro*. ‡ One of these pigs sickened and died. Necropsy lesions were not those commonly associated with hog cholera. § One pig was given only 75 cc.

Bacteriology: Mixed bacterial growth was obtained from pigs that died or were killed when moribund. *Pasteurella multocida* was obtained from 6 of 9 such pigs. No *Salmonella choleraesuis* was obtained.

vivo. Testing of vv 4081 for deviant characteristics after it has had numerous serial passages in the pig may determine its stability.

As the use of virulent virus continues to be replaced by the modified live virus as the method of controlling hog cholera, the chances of virulent variant virus appearing under field conditions are greatly reduced. Even though it may be possible for an immunological variant to develop from the modified live virus, it would seem that it would go unrecognized if it retains its form of lacking virulence. However, until the means is made known by which immunological variants within a type arise, those areas where virulent hog cholera virus exists constitute a possible hazard.

The problem of different immunological types and immunological variants within types is one of great practical importance in disease control. It is imperative to recognize this problem in a disease such as foot-and-mouth disease, especially in those countries using vaccination as a part of their control measures. Whenever a new variant appears, there is always the question of whether the vaccine for control should be prepared from the variant or from a strain or strains previously used. Available evidence suggests that new foot-and-mouth disease variants originate in the field, and it is frequently considered that they have their origin in vaccinated animals. The evidence supports the view that the variants within a type appear as a pure strain and not as a mixture of an old and a new strain. The presence of these variants has provoked much thought regarding possible lines of experimental studies that might demonstrate the means by which new types, or at least variants within types, appear or are brought into existence. In 1956, this subject was discussed in a meeting of a joint committee of the Food and Agriculture Organization and the International Office of Epizootics.[2, 14]

Some aspects of hog cholera may make it a preferred disease for investigations of the subject of induction of immunological variants. One favorable characteristic is that this disease occurs only in swine. Consequently, there is every reason to believe that the immunological variant described here had its origin in swine in 1949. There is great doubt that it had its origin in a vaccinated animal, since much effort is made to use only cholera-susceptible pigs for the production of simultaneous virus. Since the variant was found in simultaneous virus, there is evidence to support the view that the simultaneous viruses responsible for the losses in 1949 and 1950 were mixtures of regular and variant viruses. Although the results presented in this report are not conclusive evidence that this variant virus has now been obtained in a pure, antigenically distinct form, it is possible that further passages may indicate that it has been.

Epizootics of hog cholera associated with forms of variant virus have been reported in Morocco [11, 17] and in France.[9, 10]

The variant virus encountered in France was obtained from field cases and not from simultaneous virus. It remained stable, and apparently no difficulty was experienced with it in preparing satisfactory CVG vaccines. Recognition of immunological differences of various strains of hog cholera viruses led to discussions in 1954 by the International Office of Epizootics of recommendations for comparative studies of these agents.

SUMMARY

Whether procedures could be developed whereby the immunological variant of hog cholera virus responsible for the losses of swine in the Midwest in 1949 could be isolated was investigated. The methods used proceeded from the assumption that the simultaneous virus used in vaccination was a mixture of regular and variant viruses. Since current studies had shown that regular virus could be propagated in tissue culture medium, it seemed possible that this method, with modifications, might also be used to separate the variant virus in a stable form.

Previous investigations had shown that it was necessary to make serial passages of the variant virus in swine simultaneously with a subprotective dose of serum in order to maintain a virus capable of showing immunological variation.

Earlier work had shown that the mixture of regular virus and variant virus retained variant characteristics after 16 passages in swine; the first nine passages simultaneously with subprotective doses of serum and the last seven without serum. However, the variant characteristics were lost after seven more serial passages with-

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out serum. Consequently, starting with the sixteenth passage, 12 additional passages were made. The first seven passages were made with serum and the last five without serum, making a total of 28 passages. When tests in swine showed that the twenty sixth passage had characteristics of variant virus, the twenty eighth passage was used as inoculum for the first passage in tissue culture. The tenth serial passage in tissue culture when tested in swine still retained its variant characteristics.

If the virus continues to maintain its stability under these procedures, it will provide new approaches to viral studies of hog cholera.

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Studies on Myoclonia Congenita. III. Drugs and Other Factors Affecting Severity of Tremor in Pigs

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PREVIOUS PAPERS [8,17] have dealt with field investigations and pathology studies of a disease of pigs which usually manifests itself by a congenital tremor. Much of the work on tremor [4,14] has been done on animals which manifest a tremor as a result of strategically placed surgical lesions in the brain. In pigs affected with myoclonia congenita, there exists a fairly large source of experimental animals which show a spontaneous tremor. Furthermore, the young pig lends itself quite well to observation and experimental study.

In these experiments, various drugs were tried on both affected and control pigs. The results of these trials and the observations on untreated animals are included in this paper.

Payen and Fournier [12] have reported the unsuccessful use of calcium phosphate, potassium bromide, powdered areca nut, cod liver oil, calcium gluconate, thyroid extracts, parathyroid extracts, uroformine, strychnine, and arsenic for treatment of trembling pigs.

Hughes and Hinman [9] reported the unsuccessful use of calcium gluconate and potassium iodide in two litters of trembling pigs. Knilans [11] tells of 2 trembling pigs being treated with anti-hog cholera serum. One pig showed almost immediate recovery, whereas tremor persisted for several weeks in the second pig.

Florio *et al.*[6] felt that they had obtained favorable results with the oral use of a synthetic antithyroid drug (amino-thiazole) in 3 trembling pigs. The same authors tried an anti-parkinsonian drug (diethamine-2-ethyl-N-dibenzo-parathiazine) on trembling pigs without beneficial results.

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MATERIALS AND METHODS

All pigs used in this study were obtained either from owners at various points in the state or from litters farrowed at the College of Veterinary Medicine. About 1,000 pigs with tremor came under general observation and, of this number, about 150 were studied rather closely.

Observations were made on three groups of pigs. Group 1 was composed of normal pigs having no history of tremor, group 2 pigs showed tremor at the time of experiment, and group 3 was made up of recovered, outwardly normal pigs with a history of tremor. Most of the pigs used were less than 1 month old. The routine procedure was that of administering a given drug and observing and recording the effects. Recording of tremor was carried out by placing each pig on a hinged platform device which mechanically transmitted the oscillations to an ink-writing kymograph.

RESULTS

CHARACTERIZATION OF THE TREMOR

Tremor activity ranged from a mild quivering, seen only in the flank region, to severe generalized involvement of the skeletal musculature. Most of the visible activity was confined to neck, limb, and tail muscles. Head movements predominated in some affected pigs and pelvic limb tremors in others. The head tremors tended to be a lateral shaking movement, best described as the sort one might use to indicate negative response to a question.

Tremor frequency recorded from the hindlimb ranged from 2 to 12 oscillations per second (graph 1). In the instances of low frequency, the use of the term "tremor" may well be questioned. Such pigs showed an extremely high amplitude oscillation. Both hindlimbs flexed almost to the point where the pelvis touched the ground before the extensor muscles contracted and the pig would again stand erect. This activity was bilateral, rhythmic, and essentially constant as long as the pig remained on its feet.

STIMULI AFFECTING SEVERITY OF TREMOR

Drinking.—Affected pigs showed marked increase in tremor activity following each

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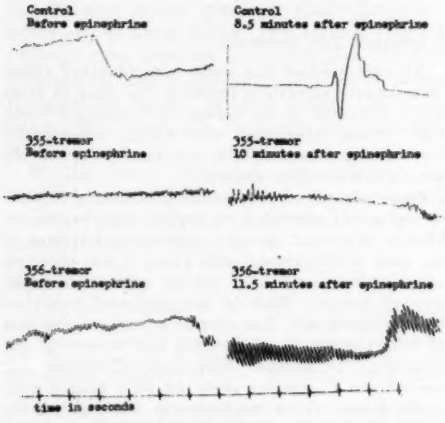
feeding of milk. Typically, they would drink rapidly for a short period and then run into a corner of the pen, where they stood huddled together as if cold, and displayed rhythmic bursts of severe tremor.



Graph 1—Kymograph records of hindlimb tremor activity. The large deflections in the control (group 1) records are due to movement. Pigs 355 and 356 are both in group 2. The effect of epinephrine was to increase the amplitude of tremor activity without markedly affecting frequency.

After a few minutes, the pigs returned to the pan of milk and the process was repeated. The signs of discomfort were especially marked if the milk was cold.

Auditory Stimuli.—In a majority of affected pigs tested, sharp sounds produced a burst of increased tremor. In quiet surroundings, a low intensity sound such as the tap of a pencil on the table top would elicit this response. Striking a metal instrument against a metal tray produced bursts of aggravated tremor much more consistently.

Excitement.—Many workers have reported that the tremor is aggravated by excitement. Pigs which showed little or no tremor while running with their littermates showed marked tremor after being placed alone in a pen for observation. Handling of these pigs, injection of drugs, or ear notching are examples of painful or excitatory stimuli which served to aggravate existing tremor.

Cold.—Glässer [7] listed as one of the characteristics of this disease the aggravation of the tremor by cold. In the course of the present experiments, the marked sensitivity of these animals to cold was frequently demonstrated and observed. Pigs with histories of tremor, but recently recovered, could be made to again show typical tremors if placed in cold surroundings. That the response was not simple shivering was indicated by the fact that control pigs of similar ages subjected to the same temperature over the same period of time showed no indication of either tremor or shivering. The production of tremor in recently recovered pigs by environmental cooling could be rapidly reversed by raising the environmental temperature to body temperature levels or above.

In animals showing frank tremor, the raising of ambient temperature may serve to reduce the severity of tremor but can not be said to serve as a cure for this disease. As a single exception to this statement, 4 baby pigs showing severe tremors were taken from a 30 to 40 F. environment and placed in a warm room (80 F.). These pigs showed complete clinical recoveries within 24 hours.

When an affected pig was placed in a sling apparatus so that it supported none of its own weight, the tremor was replaced by rhythmic bursts of what may be described as either shivering or tremor. A localized cold stimulus, such as alcohol on a small area of skin, was usually sufficient to set off the muscular activity. Room temperatures of 70 to 80 F. were maintained during these trials. In severely affected animals, these bursts of shivering in a suspended pig appeared spontaneously without a specific cold stimulus.

Rectal temperatures recorded from pigs with tremor were within normal range.

Effect of Tremor on Locomotion.—Unless the tremor was severe, it generally did not seriously interfere with locomotion. There seemed to be no associated ataxia except what may have been directly attributable to the rhythmic twitching of the limb muscles.

The low frequency-high amplitude oscillation may superimpose a jumping motion on ordinary locomotion. A few animals were unable to walk forward by use of the hindlimbs, since as soon as the pig rose to its feet it began to be propelled backward by the involuntary activity of the hindlegs.

Some of the trembling pigs showed an associated hindlimb weakness which was usually most severe shortly after the pigs were born. Gradual improvement often

occurred over a period of hours or days. Many affected pigs showed a tendency to "walk on their toes." The pastern and interphalangeal joints appeared not to be normally and fully extended.

Effect of Anesthesia.—As a part of the studies on spinal cord reflexology,[16] over 100 pigs were anesthetized (general) and studied. The drug used on most of these pigs was 5,5-diallylbarbituric acid-urethan (Dial-urethane*).

Although shivering is not unusual under anesthesia in other species, it was nevertheless constant enough in the pigs which were anesthetized to be worthy of mention. The shivering usually began in the abdominal muscles or the limbs and rapidly progressed to a generalized shivering. Since the animal was suspended with all four limbs free, the shivering was most evident in the appendages. At times, the shivering began as early as five minutes after the initial dose of the anesthetic and at other times did not appear until about one hour following the initial dose. The phenomenon was apparently associated with the depth of anesthesia since, once having appeared, it remained more or less constant throughout the experiment. Severe anesthetic depression abolished the tremor. Section of the spinal cord stopped the tremor caudal to the level of section.

The phenomenon of anesthetic tremor appeared in pigs with congenital tremors and in pigs with no histories of tremor, but was much more violent in the affected pigs. The immediate effect of the first few cubic centimeters of anesthetic was rapid cessation of any tremor which may have been present in the unanesthetized animal. However, as deeper anesthetic levels were reached, shivering appeared. There was some indication that a cold stimulus would hasten the onset of this response but, once started, the shivering continued even at the environmental temperature of 80 F., which was usually maintained as a minimum during these experiments.

Effects of Epinephrine.—Early in the course of this investigation, it was observed and reported [15] that parenterally administered epinephrine had a notable aggravating effect on tremor in affected animals.

The epinephrine used was a proprietary preparation of 1:1,000 dilution. Since it was of animal origin, it could be expected to contain a small amount of norepinephrine. The drug was administered intramuscularly in doses which varied from 0.05 to 0.15 mg. per kilogram of body weight. The pigs used ranged in age from 8 to 150 days; most were less than 2 months old.

These doses of epinephrine produced no visible effect in group 1 (control) pigs. In essence, the effect of moderate to large doses of epinephrine in affected pigs was either a marked aggravation of the existing tremor (graph 1), or a production of tremor in animals which had apparently recovered from the disease of myoclonia congenita. This response was obtained in five to 18 minutes in 25 of 29 pigs in one series of trials. The 2 mature pigs (group 3) tested did not respond to epinephrine with any indications of tremor. However, 1 sow of group 3 (recovered) developed a lameness after farrowing her second litter and again developed a tremor. She responded typically to an injection of epinephrine. A less consistent effect of epinephrine in affected animals was that of producing what seemed to be extreme discomfort. This effect was manifested by activities such as rolling over, more or less constant locomotor activity, attempts to scale the enclosure, and intermittent squealing.

During the latter part of this investigation, several litters of trembling pigs were farrowed from gilts which were bred for an entirely different experiment being conducted by another investigator. Since only a few of the total litters sired by the 1 boar contained trembling pigs, it was decided to test the nonaffected litters to determine if subclinical manifestations of the disease might exist.

A total of 59 pigs, 24 to 54 days old, from eight litters were used in this trial. Only a few animals in two of the eight litters showed visible tremor when the experiment began. Each pig was injected intramuscularly with a moderate to large dose of epinephrine hydrochloride. Time latencies for the effect of epinephrine varied from ten to 25 minutes, and within this period over 90 per cent of the 59 pigs began to show some degree of tremor. Some of these animals showed severe generalized tremor, and others showed only a slight tremor noticeable in the head or hindlimbs.

In two litters, the signs of what appeared to be discomfort were extremely

* Supplied by Ciba Pharmaceutical Products, Summit, N.J.

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marked. Some stood huddled together in a hunched position as if very cold, although the room temperature was well over 80 F. Other effects were knuckling at the pasterns, piloerection, and hindlimb weakness (inability to extend the limbs fully). As an entire group, the most marked effect of epinephrine was either aggravation of the existing tremor, or production of tremor in the pigs which had not shown it previously. Except for a few of the most severely affected animals, the effects of epinephrine had disappeared in about four hours after the injection.

Taking into account all of the trials on the visible effects of epinephrine, the duration of effect varied considerably. However, in most instances, it was a matter of hours. In a few pigs, a marked aggravation of tremor was still present 24 hours after treatment with epinephrine. In the pigs showing the prolonged effect, there were also concomitant signs of ataxia, hyperesthesia, and diarrhea. Trials now in progress on the effect of norepinephrine indicate that, in similar dosages, it does not have the tremor-aggravating effect of epinephrine.

Effects of N-(2-Chloroethyl) dibenzylamine Hydrochloride.—In order to test what part an organic epinephrine imbalance might play in the underlying mechanism of this tremor of pigs, it appeared advisable to test the effects of a sympathicolytic drug. Three pigs showing moderate tremor were treated with *N*-(2-chloroethyl) dibenzylamine hydrochloride (Dibenamine*) at dosages of 2.5 mg./kg. body weight, 5.0 mg./kg., and 10.0 mg./kg., respectively. There was a temporary alleviation of tremor in 1, but not in the other 2. There may possibly have been an aggravation of tremor in the latter 2 pigs. Doubling and tripling the dosage on these 2 pigs resulted in transient (15 to 20 minutes) alleviation, followed by severe aggravation of the tremor.

Dosages of 5, 7, 9, 10, 11, and 13 mg./kg. were administered subcutaneously in 6 pigs (one trial per pig) which were showing moderate to marked tremor about 30 minutes after treatment with epinephrine. Two of these pigs showed a temporary improvement; the other 4 did not improve visibly.

Effect of Histamine.—Subcutaneous injections of histamine diphosphate were made in 8 pigs from groups 2 and 3.

This drug produced tremor in 5 of 6 animals (group 3) where tremor was not present before the injection, and produced marked tremor increase in 2 pigs (group 2) which were showing slight tremor before the injection. The latency of this effect varied from ten to 32 minutes. These effects were seen despite the administration of 2.5 mg. of the antihistaminic drug, methapyriline hydrochloride (pyrahistine*) to each of 2 of these pigs. Except for the increased tremor activity, none of these pigs showed the restlessness and signs of extreme discomfort which were evident when 5 of them had been treated with epinephrine a week previously.

DISCUSSION

Bishop *et al.*,[1] in a rather extensive discussion on tremor, refer to the seeming proneness of the spinal level to express itself in a pattern of clonic tremor. Therefore, they feel that a number of factors (variations in the influence of higher centers) may permit the release of this clonic tendency.

With respect to one of the physiological hyperkinetic phenomena, Perkins[13] has concluded that the rhythmicity and rate of shivering depend on a mechanism which involves the proprioceptors and resembles that of a reflex clonus. However, his experiments involve deafferentated segments of the spinal cord, and there appears to be no experimental differentiation between the proprioceptive and exteroceptive effects. Burton and Bronk[3] have reported the effects of posture on the shivering response in cats. Apparently the effect is one of facilitation upon stretching of the muscle.

Several aspects of the observations on these trembling pigs suggest either the presence of an abnormally low shivering threshold or at least a remarkable synergism between the basic cause of the tremor and cold stimuli. Lowering the environmental temperature, ingestion of liquids of below body temperature, and cooling of the body surface with water were all stimuli which served to severely aggravate the tremor in affected animals.

Pigs under anesthesia appear to be un-

* Dibenamine is produced by Merck, Sharp, and Dohme, West Point, Pa.

* Pyrahistine is produced by Pitman-Moore Co., Division of Allied Laboratories, Inc., Indianapolis, Ind.

usually susceptible to cold stimuli with respect to initiation of shivering. Cort and McCance [5] have produced shivering in the lightly anesthetized pig by admitting room air directly into the trachea. This shivering was found to continue (even if the inspired air was warmed) until body temperature of the pig was raised from 39.0 to 39.5 C. This anesthetic shivering was almost invariably present in the pigs used for our experiments on spinal reflexes. The degree of shivering appeared to depend on the level of anesthesia, but was far more marked in group 2 animals than in either control or recovered pigs. The fact that the shivering was abolished caudal to the level of spinal cord section also accords with the findings of Cort and McCance.[5]

Bishop *et al.*[1] have published records of tremor produced by the injection of epinephrine in man. King *et al.*[10] have also observed tremor to result in man from the administration of epinephrine. Considering the above findings and the tendency of tremor to increase under excitement, it seems reasonable that epinephrine should exert an aggravating effect on tremor in swine also.

Both the scale and the results of the trials with *N*-2(chloroethyl) dibenzylamine hydrochloride allow no conclusions to be drawn. However, further trials on the effects of sympathicolytic drugs are indicated.

Since histamine is known to cause a release of epinephrine from the adrenal medulla,[2] its effect on tremor activity could be simply an indirect one. In fact, many of the stimuli which produced increased tremor activity could owe their effect to epinephrine release from the adrenal medulla. For example, cold and excitement are known to stimulate release of epinephrine in animals. The exact mechanism by which the epinephrine acts can not be determined with the evidence available.

SUMMARY

Observations made on approximately 1,000 pigs affected with myoclonia congenita were described. These observations were made on both intact and anesthetized animals. The results of a large number of trials indicated that parenterally administered epinephrine will markedly aggravate existing tremor and in many instances produce a temporary resumption of tremor in recovered pigs. These effects were observable for as long as 24 hours in some pigs. Subclinical cases of myoclonia congenita were demonstrated by the injection of epinephrine. Effects of the administration of other drugs were also reported.

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Embryonal Nephroma in Swine

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Most of the recent work on neoplastic diseases of food-producing animals has been concerned with general surveys or with descriptions of the more common tumors. However, there has been no recent report in English of a large series of embryonal nephromas, although they are found frequently in swine. Because of this, and because their similarity to Wilms's tumor in children makes them important from a comparative point of view, a re-examination of all cases of this tumor in the files of the Animal Disease Research Laboratory, Denver, Colo., was undertaken as a continuation of the study of tumors in food-producing animals.

Several things were desired in this study. These included a precise morphological description of the tumor, based on the examination of a large number of individual cases. Data on the inclusion of such tissues as bone, cartilage, smooth muscle, and skeletal muscle were also sought. Observations were made on the age and sex of swine in which embryonal nephroma, as well as other porcine tumors, occurred. Finally, an attempt was made to correlate the gross and histological findings of the porcine embryonal nephroma to those reported for Wilms's tumor, and to compare the tumor in swine with similar tumors of other food-producing animals.

Review of Literature

The early reports on embryonal nephroma were limited to cases in man. According to Willis,[24] Eberth, Cohnheim, Marchand, Eve, and Williams were among the first to describe it. Day [5] first called attention to this tumor in animals in 1907. His report was based on a series of eight porcine neoplasms, all of which originated in federally inspected slaughterhouses. Packinghouse material was also utilized by Davis *et al.*,[2] who found 15 nephromas in 26 porcine tumors and one in 90 bovine tumors, and by Monlux *et al.*[27] The latter authors collected all bovine, ovine, and porcine tumors diagnosed in the course of routine postmortem examination at federally inspected packinghouses in Denver, Colo., during the years 1953 and 1954. They found 13 porcine embryonal nephromas among the 782,700 hogs slaughtered during this period.

Much of the literature on this tumor in animals is the work of Feldman.[6–11] In one study, he found nine nephromas in 135 porcine neoplasms. In another, he collected 11 tumors over a 5-year period. Seven of these were purely epithelial, the remaining four being of the mixed type. He also reported extranephric lesions in a hog and in a sheep. In addition to these, he considered a series of 46 nephromas in his monograph.

In an extensive survey of the literature, Flir [13] found the embryonal nephroma reported 106 times in animals: 88 in swine, four in horses, five in cattle, two, or possibly three, in sheep, and four in dogs. He also reported two cases of his own—one in a pig and one in a rabbit.

Jackson [20] mentioned ten examples of this tumor—five in fowls, two each in horses and cattle, and one in a sheep. He stated that not a single case of embryonal nephroma in swine had been encountered in South Africa. Smith and Jones [29] recorded 59 embryonal nephromas from various animal species. Swine accounted for 44 of their cases; cattle for eight; dogs, horses, and sheep for two each; and a cat for one.

In other reports of this tumor in food-producing animals, László [23] found one nephroma in 60 swine tumors, and deMenezes [4] found one in 113 bovine tumors.

The nephroma is thought by some to occur more frequently in chickens than in mammals.[12] Guérin [16] assembled 42 avian cases from the literature (including Jackson's five cases) and added four of his own. Hoogland [18] reported three cases in chickens.

Greene [15] reported 13 cases in rabbits—nine from the literature and four which he had found in the course of over 6,000 necropsies.

The rest of the nephromas found in the literature appeared as reports of individual cases. Keller [22] found one in a 9-month-old hog; Tesseraux and Mayer,[30] one in a 4-week-old calf; and Walker [31] found an extranephric nephroma in a calf. Cases in dogs have been reported by Jones,[21] Medway and Nielsen,[25] Savage and Isa,[28] and Weitz and McClelland.[33] Guérin [16] reported one case in a rat.

Materials

The material on which this study was based is contained in the files of the Animal Disease Research Laboratory, Denver, Colo. It consists of records and histological sections from specimens

From the Animal Disease Research Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Denver, Colo.

which were submitted by veterinarians engaged in meat-inspection activities at federally inspected packinghouses throughout the United States. This material was submitted for histological diagnosis to the division's pathological laboratories of the U.S. Department of Agriculture. It consisted, in part, of reports from 164 histologically proved cases from the Chicago laboratory, 60 of which were complemented by stained sections.

These reports for which no slides were available are important in themselves, since some of them were used by Day [8] in his original paper and have historical significance. The 60 cases in which slides were available were all in swine. In addition, 57 porcine tumors from the Denver laboratory were used. These cases were all complete, consisting of both reports and sections. The above material was supplemented by eight porcine tumors collected during the course of the study, and by two others—one ovine and one bovine tumor.

Thus, there were available for microscopic examination a total of 125 porcine tumors, one bovine tumor, and one ovine tumor. A total of 229 case reports were examined.

OBSERVATIONS

The cases were grouped according to the age and sex of the animals, the number of tumors in individual animals, the presence or absence of metastasis, and the state of origin. The approximate age of the animal was stated in 172 of the 229 reports of porcine nephromas; 139 were found in animals under 1 year old, 26 in animals between 1 and 2 years old, and 7 in animals over 2 years old. Tumors were found 106 times in females and 54 times in males; in 69 cases, the sex was not stated. Single lesions were found 153 times and multiple lesions 66 times, with most of the multiple lesions being bilateral. In ten instances, the number of tumors found was not stated. Metastasis was reported in nine cases, four involved the lung, two the lung and liver, and two the lung, liver, and one of the lymphoid organs; in one case the metastasis was generalized. Most of the tumors examined originated in Illinois and in Colorado, 101 and 41 cases, respectively. This apparent preponderance resulted from the nearness of the laboratories to the large packing centers in these states. Only three other states were represented by more than ten cases; Ohio had 17, Minnesota 16, and Iowa 13. The remaining 41 cases originated in 13 states, with a geographical range from California (5) to Tennessee (1).

Several things became apparent on examination of these data. The embryonal

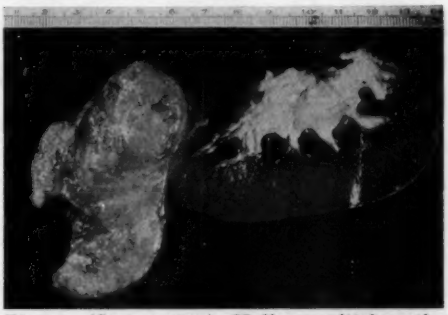

Fig. 1—(Case 133900) Median sagittal section of a kidney with a medium-sized nephroma at one pole. Trabeculae can be seen with difficulty but the capsule is readily distinguishable, especially at the border of the neoplasm and the adjacent renal parenchyma.

nephroma was shown to be a tumor of younger animals, the majority originating in states where the hog raising industry is highly developed and where hogs are usually marketed at about 6 months of age. Also, the tumor is usually a single lesion with little tendency to metastasis. In this series, the tumor occurred approximately twice as frequently in the female as in the male.

There was one point of variance from generally accepted concepts. Although this tumor has been considered by many to be the most frequent neoplastic disease of swine, only 220 of the 941 recorded porcine tumors in the collection were embryonal nephromas, while 473 were malignant lymphomas. Thus, the nephroma accounted for 23.4 per cent, and the malignant lymphoma for 50.3 per cent of the total.

Fig. 2—(Case 133878) A small pedunculated tumor protruding from the kidney. The capsule and trabeculae are especially prominent. Gömöri's trichrome; x 3.

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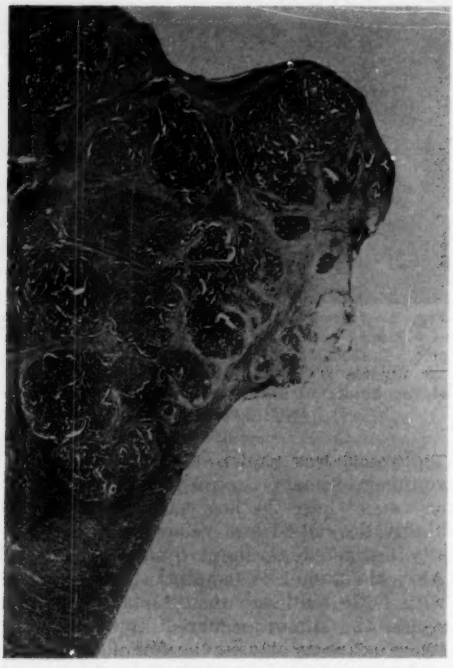

Fig. 3—(Case 132176) An embryonal nephroma expanding within the cortical parenchyma of the affected kidney. Compression of the normal renal tissue has occurred (arrow). H & E stain; x 3.

Grossly, the embryonal nephroma is a firm, light colored, lobulated tumor, with a dense connective tissue capsule and abundant trabeculae. The trabeculae may be difficult to discern in fresh specimens, but the capsule can usually be recognized readily. The tumor is fleshy, and the cut surface frequently presents a white, lardaceous appearance (fig. 1). The smaller specimens are usually of the same color and consistency throughout, whereas the larger ones may contain foci of necrosis, with consequent discoloration of the adjacent tissue. Foci of hemorrhage and areas of bloody discoloration are not uncommon. The nephroma does not appear to be invasive on gross examination, but the larger tumors frequently compress the adjacent renal parenchyma, and occasionally leave only a narrow rim of functional kidney tissue.

Although extranephric and metastatic lesions have been recognized, the kidney is the characteristic site of origin. They most commonly arise at one pole of the affected

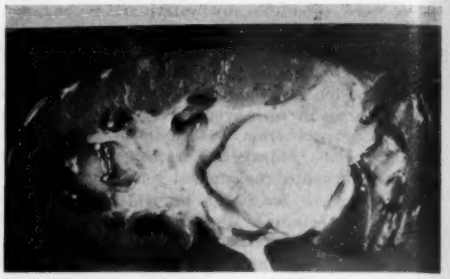

Fig. 4—(Case 133902) An embryonal nephroma which has penetrated from its point of origin in the cortex to the renal pelvis.

kidney, although it is not unusual to find lesions opposite the hilus. Generally, only a single tumor will be found in one or possibly both kidneys. However, in cases of multicentric neoplasia, an entire organ may be dotted with small tumors. All of the neoplasms in this series appeared to originate in the subcapsular cortex of the kidney. Subsequent growth took one of three basic forms. The tumors grew out from the kidney as a pedunculated growth (fig. 2), they expanded in the cortical parenchyma with resultant compression of the renal tissue (fig. 3), or they grew by expansion beyond the limits of the cortex and penetrated the medulla. In some instances, the tumor penetrated not only the medulla, but the renal pelvis and ureter as well (fig. 4).

The variations in size were tremendous. The smallest example in this collection was approximately 3 mm. in diameter and was found in a case of multicentric neoplasia. The largest was a single tumor 60.0 cm. in diameter. Most of the neoplasms measured from 2.0 x 3.5 cm. to 17.0 x 30.0 cm. Several were described by weight. Day [3] reported one instance in which the tumor weighed 60.0 lb. His original case, included in this study, was the largest in the series. Other large specimens, weighing 40.0, 37.5, 35.0, 25.0, and 20.0 lb., were also described, together with several smaller ones of approximately 5 lb. Two cases, in which both weight and dimensions were reported, are included for comparative purposes. One tumor weighed 35.0 lb. and measured 40.0 x 28.0 x 10.0 cm.; the other weighed 10.0 lb. and measured 25.0 x 20.0 x 15.0 cm.

An outstanding microscopic characteristic of the embryonal nephroma was uni-

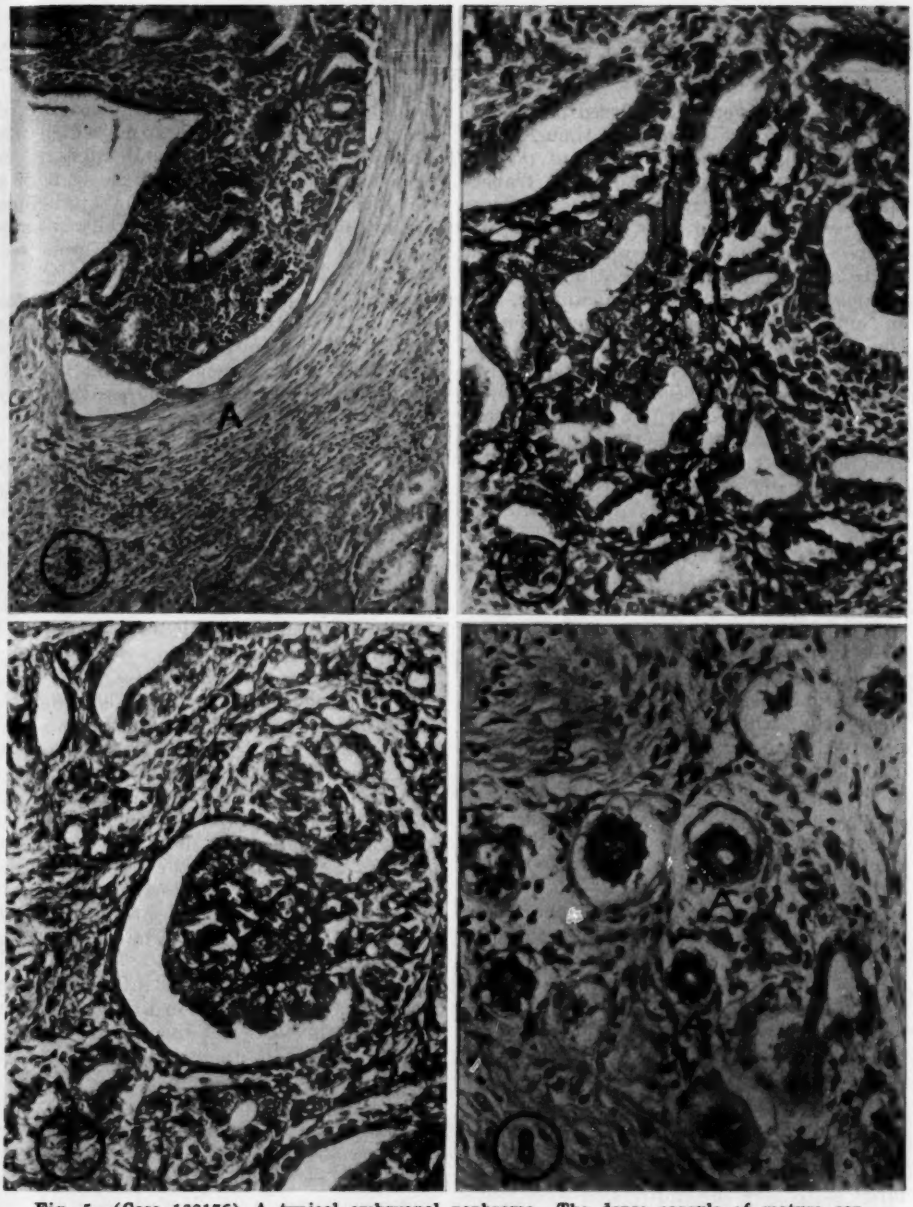

Fig. 5—(Case 132176) A typical embryonal nephroma. The dense capsule of mature connective tissue (A) separates the neoplasm (upper left) from the normal kidney (lower right). Tubule-like epithelial structures (B) are surrounded by relatively undifferentiated embryonic connective tissue (C). H & E stain; x 110.

Fig. 6—(Case D 57) Basement membrane around well-developed tubules in an embryonal nephroma. Most of the tubules in this specimen are composed of a single layer of quite mature cells. The sarcomatous element (A) is also fairly well differentiated. The cells of this component are somewhat elongated, but their nuclei remain plump. PAS technique; x 225.

Fig. 7—(Case D 57) A papillary structure in an embryonal nephroma. These structures apparently represent a longitudinal section through a tortuous tubule. The flattened epithelial lining of the structure is continuous with an epithelium having a more characteristic appearance (A). The concavity of the structure contains well-developed tubules with distinct basement membranes. These tubules are cut in cross section. PAS technique; x 225.

Fig. 8—(Case 12969) An embryonal nephroma in which the epithelial element (A) is well differentiated and the connective tissue component (B) is poorly differentiated. H & E stain; x 225.

formity. They were all enclosed in a dense capsule of mature connective tissue which separated them from the adjacent normal tissue, and also formed the many trabeculae which were present. The neoplasm itself was composed of two distinct elements—an epithelial component which tended to form tubules that imperfectly mimicked those of the normal kidney, and an embryonic connective tissue element (fig. 5). An adequate blood supply was provided by relatively normal vessels in the capsule and trabeculae, and by rudimetary, thin-walled structures in the connective tissue element of the tumor. The growths were composed of cells of varying degrees of differentiation. In their most immature form, the cells of the two elements were difficult to distinguish from each other. It was necessary in such cases to classify these undifferentiated (blast) cells on the basis of the more mature forms with which they were associated. Transitions from these primitive types to distinguishable epithelial or connective tissue cells could usually be recognized. The blast cells, which appeared to be pluripotential, had large, vesicular nuclei, fine dustlike chromatin granules, and usually at least two nucleoli. The cytoplasm was scanty, basophilic, and barely visible.

As differentiation took place, the epithelial cells developed smaller, less vesicular nuclei and the number of nucleoli diminished. The cytoplasm become more abundant and changed from basophilic to acidophilic. The round or oval epithelial cells became cuboidal or columnar, and the nuclei assumed a basal position in the cell. The immature epithelial tissues appeared as sheets or nests of cells and, as differentiation progressed, tubules formed. The earliest tubules consisted of a concentric arrangement of oval to cuboidal cells. These were usually solid, but a lumen soon appeared. The lumen sometimes became so large as to give the impression of a cystic space, and it sometimes contained hyaline material. The walls of the tubules consisted of a single layer of well-differentiated cells, or of two to six or seven layers of immature cells. The well-developed tubules usually had a distinct basement membrane, which could be demonstrated by the PAS stain technique[24] (fig. 6). They were usually tortuous and occasionally formed papillary structures which had some resemblance to glomeruli. The papillary structures usually consisted of an arched, hemispherical space which was lined by epithelial tissue and appeared to be a longitudinal section of the wall of a tortuous tubule in which the epithelium became flattened. Their concavity usually contained other tubules cut in cross section (fig. 7).

The differentiation of the cells of the sarcomatous element was somewhat similar to that of the epithelial component. The architectural arrangement was at first extremely loose, becoming progressively more compact, although it never approached the degree of density shown by the connective tissue of the capsule and trabeculae. As the cells matured, they tended to elongate and exhibit more cytoplasm than was visible in the blast cells. However, the nuclei remained oval, and the cytoplasm tended to remain slightly basophilic. This sarcomatous tissue was found dispersed in the spaces between the epithelial structures of the tumor.

Examination of stained sections from 125 porcine cases revealed considerable variation in the proportion of the two components and in their degree of differentiation. In 93 cases, the epithelial element predominated, while the sarcomatous element was most prominent in 17, and the two components were about equally prevalent in 15 instances. The epithelial component was poorly differentiated in 64 cases, fairly mature in 44, and of an intermediate degree in 17. The sarcomatous element was primitive in 87 cases, well differentiated in 24, and intermediate in 14. It was not uncommon to find examples in which the two components varied markedly in degree of differentiation (fig. 8, 9).

There did not seem to be any relationship between mitotic activity in the individual tumors and metastasis. Mitotic activity was considered intense in 70 cases, moderate in 18, and slight in 13; it was not recognized in 24. Metastases were seen in only four of the complete cases. The lungs were involved in three of these, with the liver and the abdominal wall additionally affected in one and the liver and lymph nodes in another. The fourth metastatic lesion consisted of tumor implants on the peritoneal surface of the liver (fig. 10). Mitotic activity was considered intense in two of these cases, moderate in one, and slight in one. No histological evidence of

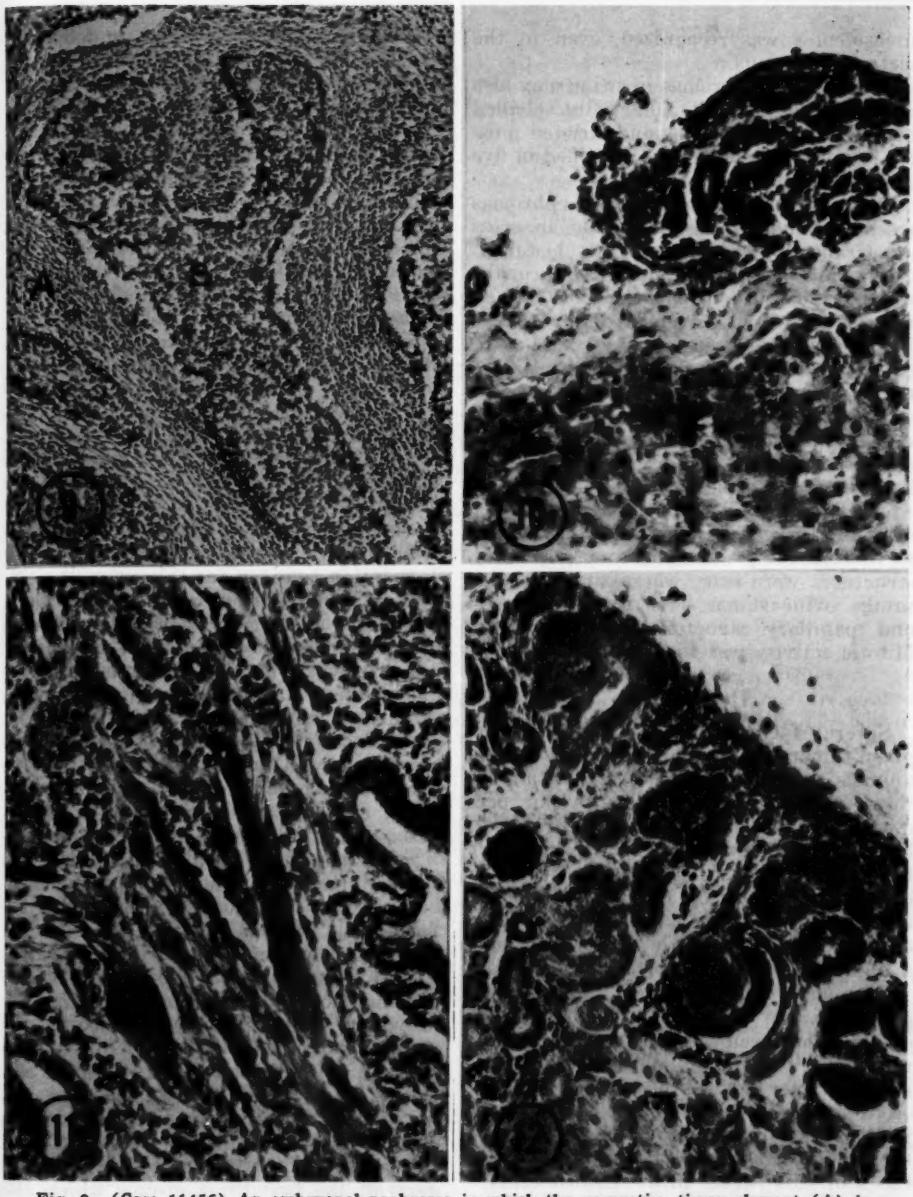

Fig. 9—(Case 11456) An embryonal nephroma in which the connective tissue element (A) is well differentiated and the epithelial component (B) is poorly differentiated. H & E stain; x 110.

Fig. 10—(Case D 3009) Tumor implant on the peritoneal surface of the liver. The predominantly epithelial character of the metastasis is obvious. H & E stain; x 225.

Fig. 11—(Case 15893) Striated muscle fibers in embryonal nephroma. Gömöri's trichrome; x 225.

Fig. 12—Developing kidney in a 17.5-cm. pig fetus. The similarity between the appearance of the outer cortical zone and that of an embryonal nephroma is striking. H & E stain; x 225.

invasiveness was recognized, even in the metastatic lesions.

Embryonal nephromas in swine may also contain other elements. Partially calcified bone was present in one, and striated muscle (fig. 11) was definitely identified in five of the tumors in this series.

The nonporcine embryonal nephromas resembled the neoplasm in swine in gross appearance, but differed slightly histologically. Both were single, unilateral growths which were well encapsulated and showed no evidence of invasiveness or metastasis. The bovine tumor measured 20 x 11 x 11 cm., but the information as to the size of the ovine tumor was lacking. Microscopic examination revealed that the sarcomatous element predominated in both cases. The epithelial element was well differentiated in both, and formed rather long tubules which were usually lined by a single layer of cells. In the bovine tumor, the tubules were relatively straight and papillary structures were rare, whereas the tubules in the ovine tumor were rather tortuous and papillary structures were common. Mitotic activity was slight in both cases.

DISCUSSION

Several factors make comparison of a given tumor in man and in food-producing animals difficult. The most important of these is that most animals are not permitted to live out their normal life span, but are slaughtered while in the prime of life. Also, adequate necropsies are infrequently performed on food-producing animals that die before marketing, especially on swine and chickens, both of which have a high incidence of embryonal nephroma.

The embryonal nephroma, which is regarded as the commonest renal tumor of children, has been the subject of numerous reports.[1, 5, 13, 14, 17, 26, 32] Although approximately 65 per cent of these tumors in man occur before the third year of life, and 75 per cent before the fifth year, they have been reported as late as the eighth decade.[5] Cases of this tumor in a 7-month fetus [13] and in a child, 1 month old,[14] have been reported. The tumor in man is usually unilateral and has no sex predilection. The age incidence in swine is somewhat similar, with 77 per cent of the tumors in our series occurring during the first year of life. The oldest animal in the series was a 6-year-old sow. No reported cases of this tumor in very young pigs were found, a deficiency which Flir [13] also noticed. The porcine tumor is usually unilateral, as it is in man. The tumors in this series occurred approximately twice as frequently in the female as in the male, which differs from the usually recorded incidence in human beings and in swine.

The porcine embryonal nephroma does not differ from the human tumor as described in the literature. Both appear as firm, lobulated growths, which are well encapsulated and light in color. They both consist of a combination of neoplastic glandular and connective tissues. Other components, including smooth muscle, skeletal muscle, cartilage, and bone, have been found in these tumors in man. Cartilage was not found in any of the tumors of this series, nor was smooth muscle positively identified. The epithelial element usually predominates in the porcine tumor, but the cells of both components conform to the description as given for the tumor in man. The papillary structures (fig. 7), which occur in both human and animal tumors, have been variously designated, usually by names which connote a relationship to the glomerulus. However, in swine, they are usually composed of neoplastic epithelial cells, and rarely have any blood vessels associated with them. Because of this, we prefer to designate them as papillary structures, rather than by a name which suggests a relationship to the glomerulus.

Several attempts have been made to classify the nephroma on a histological basis. This is difficult because of the differences in the amount of epithelial and connective tissue elements in individual tumors. They have been divided into two extreme types,[26] those composed primarily of young, undifferentiated connective tissue and those composed chiefly of a similar type of epithelial tissue. They have also been classified as:[13] tumors of homogeneous epithelial structure; mixed celled forms with epithelial and connective tissue components; rhabdomyoadenosarcoma; and tumors of the first three groups which also contain supporting tissue substances.

None of these methods is entirely satisfactory, and it is doubtful whether any adequate classification can be made on such a basis. Attempts of this kind have resulted in the appearance of such designations as adenosarcoma, adenomyosarcoma, sarcocarcinoma, and rhabdomyoadenosarcoma, all

of which are self-contradictory. Deming[5] thought that these tumors should be classified simply as "nephroma," because they contain all the elements of the kidney.

Many theories have been advanced to explain the histogenesis of these tumors. Deming[5] gives a rather complete list of the various theories, together with their protagonists. One widely accepted concept is that these tumors arise as the result of persistence of metanephrogenic tissue in the completely formed kidney. The tumors in this series all originated in the cortex, immediately below the capsule, in an area that would correspond to the neogenic zone of Huber.[19] The development of the tubule-like structures in the nephroma resembles that described for the normal uriniferous tubules. Moreover, the appearance of the neogenic zone in a 17.5-cm. pig fetus (fig. 12) was strikingly similar to that of representative embryonal nephromas. All of the foregoing supports the contention that these tumors arise as the result of remnants of metanephrogenic tissue in the completely developed kidney.

The tumor in man is considered malignant, and although there is not complete agreement as to when metastasis may be expected, it is agreed that it will occur eventually. The human tumor is locally invasive and rapidly growing. These conditions lead to the production of local extrarenal lesions and to distant metastasis, either through bursting of the capsule or through blood vessel invasion. The tumor may invade the renal vein, the vena cava, or the lymphatics. Metastases are most commonly found in the lungs, with the liver and the lymph nodes taking a secondary place. The tumor in swine metastasizes rarely, but when it does, it affects the same organs as in man. The porcine tumor is a rapidly growing neoplasm, as shown by the large size it attains in young animals, and by the frequency of mitotic figures in the lesions. However, none of the tumors in this series showed any evidence of invasiveness, either grossly or histologically. Grossly recognizable invasion of blood or lymphatic vessels apparently has never been recorded in animals. Histologically, the porcine tumors studied were all well encapsulated, even in cases of metastasis. The question of whether these tumors would become obviously malignant if the animal lived could not be resolved by this investigation.

SUMMARY

All cases of embryonal nephroma in the files of the Animal Disease Research Laboratory at Denver, Colo. were re-examined as a continuation of the study of tumors in food-producing animals. A total of 229 case reports were studied. In 125 porcine cases, one bovine case, and one ovine case the reports were complemented by stained sections.

In swine cases in which the age was recorded, 77 per cent of the tumors were in animals under 1 year of age, 15 per cent in animals between 1 and 2 years old, and the remainder in animals over 2 years old. The oldest was a 6-year-old sow.

The sex ratio was approximately 2 to 1; 106 females and 54 males. Sex was not recorded in 69 cases.

Single lesions occurred 153 times; multiple lesions 66 times.

Metastasis was found in nine cases.

The specimens varied in size and in detailed microscopic structure, but were otherwise remarkably similar. The embryonal nephroma can be described as a firm, lobulated, light-colored growth in the kidney, which had a distinct capsule and numerous trabeculae of dense mature connective tissue. Microscopically, they were composed of epithelial and connective tissue components, both of which had an embryonic appearance. The epithelial component predominated in the porcine tumors; the connective tissue element predominated in both the bovine and ovine tumors. Variations in the quantity and in the degree of differentiation of both elements were marked.

The embryonal nephroma in animals is a rapidly growing tumor, which frequently shows intense mitotic activity, but rarely metastasizes. Evidence of invasiveness was lacking in all the tumors of this series.

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The Effect of Intramuscular Injections of Vitamin B_{12} on Blood Serum Levels in Swine

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VITAMIN B_{12} LEVELS of swine whole blood [3] and blood serum [4, 9, 23] have been reported. However, the contemporary trend toward the feeding of all-vegetable diets suggests the possible existence in swine of a nutritional status similar to that in human "vegans." [7, 17, 21, 22] This would invalidate any general survey of swine serum vitamin B_{12} levels to determine a normal range.

The observations of Mollin and Ross [16] on the effects of intramuscular vitamin B_{12} injections in human pernicious anemia patients suggested a physiological mechanism for the regulation of normal serum B_{12} levels. They found that total serum vitamin B_{12} concentrations rose to a maximum within one to three hours after an injection, then fell within 24 hours (if the dose was not too large) to normal levels which were maintained for at least 48 hours. Several repetitive injections given to the same patient at 48-hour intervals did not influence the 24- to 48-hour postinjection serum levels.

Demonstration in swine of the existence of a mechanism that regulates serum vitamin B_{12} levels would be of interest. A sustained postinjection plateau, if present, would tend to have clinical implications.

EXPERIMENTAL PROCEDURE

Nine crossbred, nonpregnant sows were selected at random from two Central Experimental Farm herds. They had been fed a commercial sow diet that contained a calculated 3.77 μg. of vitamin B_{12} per pound, and this was continued during the experimental period.

Each sow was given, intramuscularly, 250 μg. of vitamin B_{12} in aqueous solution. Seven of the sows were given a second injection two weeks after the first. Blood samples were removed from the vessels near the thoracic inlet immediately prior to the injection and at 1, 3, 6, 8, 24, 48 and 336 hours after the injection.

Blood serums were stored in a deep freeze. Individual serums were assayed for total vitamin B_{12} by the *Euglena gracilis* var. *bacillaris* method previously reported.[4] All serums were assayed at two dilutions on each of at least two different assay days. Each dilution was set up in quadruplicate assay tubes. There was good within and between assay agreement (mean difference between duplicates and their means, within and between assays, $\pm$ S.E. = 10% $\pm$ 1.6 and 9% $\pm$ 2.2 where n = 27 and 14 respectively).

Free serum vitamin B_{12} was determined on some of the serums by modifying the ultrafiltration method that Gregory and Holdsworth [6] used to study the binding capacity of milk. A maximum of 3.0 ml. of serum was made up to 10.0 ml. with double, glass-distilled water and ultrafiltered by means of vacuum through dialysis casing, until slightly more than 2.0 ml. of ultrafiltrate resulted. The volume of serum that was added varied with the anticipated free vitamin B_{12} content of the serum. Two milliliters of the ultrafiltrate were made up to 8.5 ml. with double, glass-distilled water, and the solution was stored overnight in the refrigerator. The residual ultrafiltrate was checked for protein with trichloroacetic acid. Only protein-free filtrates were used.

On the next morning, 2-ml. aliquots of the diluted ultrafiltrate were pipetted into four assay tubes containing 2 ml. of basal medium and 0.8 μg. of potassium cyanide per tube. The cyanide content was the same as that of individual standard tubes.[4] The test tubes were sterilized in the regular manner. All determinations were done in replicate. The dilution of individual serums between replicates was not varied in most cases, because of the low free serum vitamin B_{12} content.

This method was compared to the method of Ross [18] for determining free serum vitamin B_{12}. For the comparison, no cyanide was added to either set of assay tubes, and a separate set of standards without cyanide was prepared. This was to prevent the possibility of cyanide ions displacing some of the vitamin B_{12} bound to the serum protein, when the method of Ross was used. Final dilutions of individual serums were the same in both methods. These provisions allowed direct comparison between methods.

Combined serum vitamin B_{12} was determined indirectly by calculating the difference between total and free serum vitamin B_{12}.

From the Chemistry Division, Science Service, Canada Department of Agriculture, Ottawa, Ont. Contribution 407.

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RESULTS AND DISCUSSION

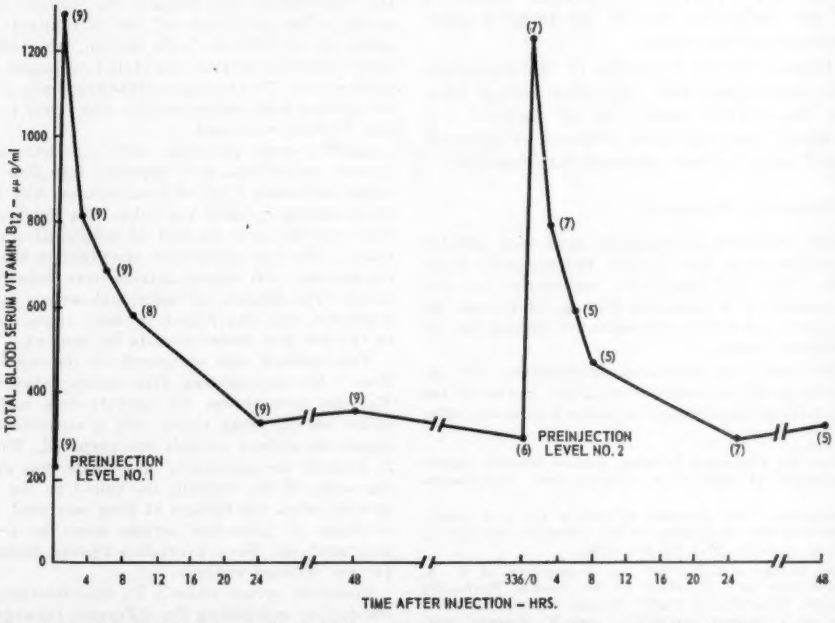
The mean, total, serum vitamin B_{12} levels of the sows prior to and following the injections are illustrated (graph 1).

The maximum, total serum vitamin B_{12} levels observed usually were found one hour after the injections. Thereafter, the levels fell precipitously until eight to 24 hours. Two-tailed, paired "t" tests showed that there were no significant differences between the 24-, 48-, and 336-hour serum levels after the first injections, or between the 24- and 48-hour levels after the second injections. Essentially, two plateaus had been produced.

To compare the effects of the first with the effects of the second injections, the means of the pooled 24- and 48-hour serum levels of individual animals after each injection were compared with each other and with the preinjection serum levels. The means of the pooled 24- and 48-hour serum levels after the first injection were significantly greater than their corresponding preinjection serum levels ($P<0.001$). There was no significant difference between the means of the pooled observations from the first and second injections.

Apparently, the amount of vitamin B_{12} from the first injection that was stored in the body, plus that in the regular dietary intake, was sufficient to maintain constant serum levels, between 24 and 336 hours after the injection, at significantly higher levels than the preinjection levels. The levels of the second plateaus were not affected by the first injections, even though the animals started with significantly higher preinjection serum levels the second time ($P<0.05$).

The rapid fall in serum vitamin B_{12} levels between one and 24 hours, and the maintenance of the plateau levels for at least 336 hours, indicate that the plateau levels are physiologically acceptable. (It has been shown in man [8, 16] and in rats [10] that the fall corresponded to the time of maximum, increased urinary excretion of vitamin B_{12}. The significance of different routes of excretion has been reported for various species.[13] Storage and utilization constitute other factors that lower serum levels.) The plateau levels do not represent a physicochemical saturation limit of the serum, although they may be considered a physiological saturation. Because



Graph 1—Effect of intramuscular injections of vitamin B_{12} on total serum vitamin B_{12} of sows fed standard commercial diets. Figures in parentheses represent the number of animals.

total serum vitamin B_{12} levels similar to the plateau levels can be attained by oral supplementation,[5] it is quite possible that the plateaus indicate normal or even optimum serum levels in swine. The findings of Mollin and Ross [16] in man are referred to again in this respect, and a similar response occurs when, for example, thiamine is injected subcutaneously.[12] It appears that there are factors which influence and possibly control, within limitations, maximum and minimum serum vitamin B_{12} levels during certain physiological states and dietary regimens.

Because it is generally believed that vitamin B_{12} influences the reproduction of various species, it was of interest to try to relate the previous reproductive histories with the various serum levels (table 1). The histories were available for only 7 of the 9 sows. The ability to farrow living young was used to separate the sows into two groups. This eliminated the influence of genetics on litter size, and the influence of external factors on offspring viability. The diet was the same during the gestation period as at the time of the injection experiment.

There was no significant difference in either the preinjection or plateau levels of the two groups, but there was a significant difference in the ratios of preinjection levels to plateau levels ($P<0.01$). Caution must be exercised in interpreting these results. However, the difference in significance, when the ratios are used, is not surprising, since there may be considerable variation in the serum response of different animals to similar prolonged oral dosing, despite the fact that the different animals may have had similar serum levels prior to dosing.[5] This presumably reflects individual variation in the balance between absorption, utilization, and excretion.

Furthermore, the ratio probably indicates nutritional status more precisely than "resting" serum levels. Mollin and Ross [15] reported that the serum vitamin B_{12} levels of normal subjects and pernicious anemia patients overlap (the respective ranges were 100 to 720 and 16 to 105 $\mu\mu$g./ml.). Thus, it would be possible for a person with a serum level in the intermediate range to be normal or deficient. If the person were deficient, then the optimum normal level would be much higher than the present, and the ratio of the two levels would be less than unity. As the degree of deficiency decreases, the ratio approaches unity. Although we still do not know what constitutes an optimum normal serum level for any individual, the plateau level does represent a physiologically acceptable (in regard to the organism), physiologically self-determined constant for any individual with which resting serum levels can be compared. The plateau may be greater than the optimum normal level, but it can never be less. Therefore the ratio can never exceed unity. Depending on the ratio between the plateau and optimum normal levels, it may not be essential to have a ratio of unity between the resting and plateau levels. This requires further elaboration, but it does not obviate the usefulness of the ratio as a criterion of nutritional status.

Certain hepatic and hematological disorders may exert influences that have to be considered. The principle is not unlike the utilization of the xanthurenic acid index to determine the vitamin B_6 requirement.[2, 14, 20]

It is of practical interest that these sows were from two large herds of different genetic backgrounds and with histories of poor reproduction and high mortality rates in their offspring. Studies have not incriminated any significant genetic, toxic, infectious, or antigenic etiological factors.

TABLE 1—Relationship Between Vitamin B_{12} Serum Levels and Reproductive Performance of Sows

Sow	Vitamin B_{12} serum concentration ($\mu\mu$g./ml.) 0 hr.	Plateau*	Ratio†	Previous reproductive performance
0941K	220	289	0.76	Produced living offspring
437J	332	442	0.75	Produced living offspring
177K	240	339	0.71	Produced living offspring
738J	188	297	0.63	Produced living offspring
Mean	245±31	342±35	0.71±0.03	
90L90L	239	421	0.57	Apparently conceived and aborted
400K	109	228	0.48	Failed to conceive during two consecutive estrous periods
0242L	126	299	0.42	Bred and aborted 50 days later
Mean	158±41	316±56	0.49±0.04	

* The plateau serum concentration is the mean of the pooled 24 and 48 hr. serum concentrations after the first injection. † Ratio $= \frac{\text{preinjection serum level}}{\text{plateau level}}$.

Although the animals were fed the same standard commercial sow diets that contained presumably adequate vitamin B_{12}, some of the sows had total serum vitamin B_{12} levels similar to 10 sows that had been fed a vitamin B_{12}-deficient, corn-soybean oil meal diet for more than two years (calculated vitamin B_{12} content of the diet was less than 1 μg./lb.). Two of the latter sows were bred and they aborted at or near term; their controls, orally dosed daily with sufficient crystalline vitamin B_{12} to produce serum levels comparable to these plateaus, farrowed live pigs.[5] Low serum levels also were found in other sows from the same two herds. Thus, contemporary supplementation appeared to be inadequate, at least for some sows.

TABLE 2—A Comparison of the Free and Combined Serum Vitamin B_{12} Determinations After Intramuscular Injection of Vitamin B_{12} Using the Methods of Ross and Ultrafiltration

		Serum vitamin B_{12} ($\mu\mu$g./ml.)				
			Ultrafiltration		Ross	
Animal	Sample	Total	Free	Combined	Free	Combined
1	1 hr.	2404	186	2218	270	2126
	3 hr.	1059	181	878	0	1059
	24 hr.	444	61	383	5	439
2	1 hr.	1168	308	860	145	1023
	3 hr.	662	159	503	22	640
	24 hr.	248	64	184	11	237
3	1 hr.	1659	419	1240	498	1161
	3 hr.	716	193	523	50	666
	24 hr.*	212	41	171	36	176

* This sample was a mixture of 24 and 48 hr. serums.

Combined serum vitamin B_{12} levels reacted similarly to the total serum vitamin B_{12} levels; the maximum levels observed occurred within one to three hours (table 2). These maximum levels were significantly higher than both the 24- to 48-hour total and combined serum vitamin B_{12} levels, when both the method described herein and Ross's method were compared. This is contrary to the findings of Mollin and Ross [16] regarding the similarly limited *in vivo* and *in vitro* combining power of serum from both pernicious anemia patients and normal human beings.

Despite the species difference, this is not surprising. Bertcher and Meyer [1] determined the *in vitro* binding capacity of human serum by using Co^{60}-vitamin B_{12} and dialysis. They found that the binding capacity increased as the vitamin concentration was raised. In addition, Sheely *et al.* [19] observed that the binding capacity of human serum was influenced by the total vitamin B_{12} content of the serum after parenteral administration of either crystalline vitamin B_{12} or liver extract.

The comparative data (table 2) show that in seven of nine comparisons, the free vitamin B_{12} measured by the ultrafiltration method is greater than that measured by the method of Ross. The ultrafiltration method removes serum protein that might[18] or might not [11] inhibit the organism. If serum protein does cause inhibition, this is especially important when trying to detect low levels of free vitamin B_{12} at low dilutions of serum. In addition, the ultrafiltration method assures that only free vitamin B_{12} is available to any assay microorganism. Thus, it overcomes the objections to methods that depend on the debatable selective ability of various test organisms to utilize only free vitamin B_{12}.

SUMMARY

Nine sows were injected intramuscularly with 250 μg. of vitamin B_{12}; 7 of them were given a second injection two weeks later.

The total and combined serum vitamin B_{12} concentrations were determined at intervals up to 336 hours after the first series of injections, and up to 48 hours after the second.

The total serum vitamin B_{12} concentrations reached peaks within one to three hours after the first injections, then fell precipitously until plateaus occurred. The plateaus were maintained from the twenty-fourth to the 336th hour. They were significantly higher than the preinjection concentrations. Similar responses followed the second injections, and the second 24- to 48-hour plateaus were identical to the first.

The combined serum vitamin B_{12} concentrations followed the same trends as were observed with total serum vitamin B_{12} concentrations.

The method of Ross and an ultrafiltration method for determining free serum vitamin B_{12} were compared. The ultrafiltration method usually gave higher values.

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Influence of Oral Intake of Vitamin B_{12} on Total Blood Serum Vitamin B_{12} Levels in Swine

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A RELATIONSHIP between oral intake of vitamin B_{12} and total vitamin B_{12} content of blood serum has been reported for man during various nutritional and physiological states.[2, 3, 9–11, 13, 14, 19, 20]

Maintenance of normal blood serum vitamin B_{12} levels is dependent on adequate intake and absorption, and freedom from certain pathological entities. Yamamoto *et al.*[21] believed that low blood serum levels may be considered a measure of poor reserve, but normal or hypernormal levels may be due sometimes to agents that temporarily release vitamin B_{12} from storage organs.

The work of Mollin and Ross [12] suggested that maximum, normal, blood serum vitamin B_{12} levels in man were regulated. The observations of Frederick [5] implied that there also were factors with a similar function in swine. The ability to absorb vitamin B_{12} also exerts an influence on serum levels; limited, normal absorption and indications of limited absorption have been reported for man [1, 7, 8, 16] and rats.[4, 18]

Because of the physiological and practical implications, it was of interest to study the relationship between different oral intakes of vitamin B_{12} and total blood serum vitamin B_{12} levels in swine. It was postulated that total blood serum vitamin B_{12} levels should vary directly with the oral intake until maximum normal levels are attained.

Experimental Procedure

In the first experiment, 6 Landrace-Chester White crossbred sows were used. They had been fed a vitamin B_{12}-deficient diet (table 1) for two years. Their serum levels had been consistently low for most of the time. They never had been bred.

Three of the sows were continued on the deficient diet; the diet of the other 3 sows was supplemented every evening for 100 days with 50 μg. of vitamin B_{12}. The dose was increased to 100 μg. per day during the next 100-day period, and to 200 μg. per day during a third 100-day period.

Extraneous vitamin B_{12} contamination was limited as much as possible.

In the second experiment, 6 gilts that had been fed regular, commercial diets were used. Three were fed the deficient diet; the other 3 were fed the deficient diet plus 300 μg. of vitamin B_{12} daily. After nine weeks, the dose was increased to 400 μg. per day for reasons of convenience.

Blood samples were taken from all animals every second week, 17 hours after the evening meal. Blood serum was assayed for total vitamin B_{12} by means of a modified [6] *Englena gracilis* method.[15]

Results and Discussion

The results of the first experiment are illustrated (graph 1). Only blood samples taken during the last 65 days of each feeding period were assayed. The mean blood serum vitamin B_{12} levels of individual treated sows during each period are shown. The mean blood serum levels of the 3 control sows during each period also are indicated. The solid line represents the relationship between the oral doses and the mean blood serum levels of the treated sows. The means were used because an analysis of variance indicated no trends in the blood serum vitamin B_{12} levels of any of the animals during any of these periods.

Regression analyses of the combined and separate data for the two groups of sows revealed that the serum vitamin B_{12} levels of the control sows showed no significant trend during the three periods. The treated sows, on the average, showed a significant increase ($P<0.01$) in the serum levels as the daily oral dose was increased from 50 to 200 μg. Tests of the quadratic component for the treated sows suggested a curvilinear response to arithmetic-dose increments ($P<0.10$), or a linear log-dose response, over the dose range used.

Because the blood serum vitamin B_{12} levels were dependent on the levels of oral intake, it is concluded that the body stores were not saturated, at least during the first two feeding periods. Partial or com-

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plete depletion of body stores was to be expected, because the sows had been fed the vitamin B_{12}-deficient diet for two years prior to this experiment, and it is also possible that the commercial diet fed prior to that time was deficient.[5] Indications of low body stores also were observed in the second experiment.

It is unlikely, in view of the factors that appear to regulate maximum, normal, blood serum vitamin B_{12} levels in swine,[5] that the levels would have risen indefinitely with increased dietary supplementation.

Although the 3 treated sows had similar blood serum vitamin B_{12} levels during the first feeding period, sow 10 responded to the oral dosing with consistently lower levels during both of the remaining two periods (graph 1). The different response illustrates the known wide variation that occurs between individuals in regard to vitamin B_{12} metabolism.

It is of interest (1) that the daily intake of sows fed contemporary, supplemented diets is calculated to be less than 50 μg./day; (2) that there was no significant difference between the blood serum levels of the control sows and those given an oral dose of 50 μg./day; (3) that 2 of the control sows were bred and aborted near term, whereas 2 treated sows (8 and 10), fed a

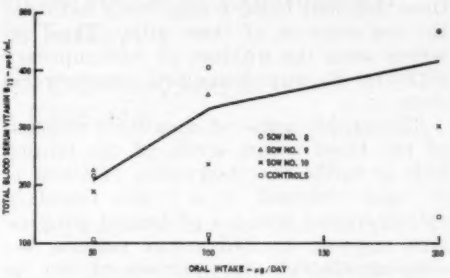
TABLE 1—Composition of the Vitamin B_{12}-Deficient Diet

Ingredients	Quantity/100 lb.*
Ground yellow corn	69.5 lb.
Soybean oil meal (solvent extracted)	10.0 lb.
Dried brewer's yeast	5.0 lb.
Vitamin-free casein	3.0 lb.
Solka-floc (cellulose) †	10.0 lb.
Ground limestone	1.35 lb.
Di-calcium phosphate	0.65 lb.
Iodized salt	0.5 lb.
Zinc carbonate	0.02 lb.
DL-methioine	50.0 Gm.
Calcium pantothenate	0.375 lb.
CALCULATED COMPOSITION	
Crude protein	15.6%
Crude fibre	12.0%
DL-methionine	0.44%
DL-lysine	0.81%
DL-tryptophane	0.18%
Cystine	0.18%
Niacin	18.9 mg./lb.
Pantothenic acid	9.0 mg./lb.
Calcium	3.0 Gm./lb.
Phosphorus	2.2 Gm./lb.
Vitamin B_{12}	<1.0 μg./lb.

Nonpregnant sows were fed 2.5 lb. of the ration twice daily, plus sufficient vitamin A and D feeding oil to meet N.R.C. requirements.

* At first the ration contained more corn and less soybean oil meal. It was changed to obviate costly lysine supplementation.

† Supplied by Brown Forest Products, Ltd., Montreal, P.Q., Canada.



Graph 1—Relationship between oral intake of vitamin B_{12} and total blood serum vitamin B_{12} levels in sows.

supplement of 300 to 400 μg. of vitamin B_{12} per day during pregnancy, farrowed live pigs. Additional pertinent observations and their possible implications are discussed in a companion paper.[5]

The effect of feeding the vitamin B_{12}-deficient diet to gilts for five and one half months, with and without vitamin B_{12} supplementation (table 2), further demonstrates the relationship between oral intake of vitamin B_{12} and blood serum vitamin B_{12} levels. The serum levels of the control gilts decreased from their original levels over a short period of time (6 to 8 weeks); those of the treated gilts responded quickly to the supplement (within 2 weeks).

Because the serum levels of the control gilts decreased to low base levels that were maintained for at least 3 months, and because these levels were similar to those of the control sows in the first experiment, it is felt that the base represents true deficiency levels for these animals. Because low blood serum levels are said to indicate low tissue reserves,[21] and in view of the indications of large tissue reserves in normal man,[17] the short time required to produce

TABLE 2—Blood Serum Vitamin B_{12} of Gilts Fed the Vitamin B_{12}-Deficient Diet with and Without Added Vitamin B_{12}

	Duration of feeding period (weeks)							
Sow	2	4	6	8	14	18	20	22
NO SUPPLEMENT								
K	158*	146	78	109	47	.....	48	66
L	163	210	.....	116	97	111	88	111
M	255	175	132	113	144	176	174	181
300–400 μG. VITAMIN B_{12} DAILY†								
N	656	627	709	760	467	522	549	443
P	729	570	753	585	532	631	697	668
Q	599	535	629	636	397	366	482	471

* Micromicrograms of vitamin B_{12} per ml. serum.

† 300 μg. vitamin B_{12} per day for the first nine weeks, then 400 μg. of vitamin B_{12} per day thereafter.

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these deficient blood serum levels indicates the low reserves of these gilts. These reserves were the product of contemporary vitamin B_{12}-supplemented, commercial diets.

The quickly achieved, maximum response of the blood serum levels of the treated gilts is unlike the observation of Chow *et al.*[3] and Chernish *et al.*,[2] who found in elderly human beings a prolonged, progressive response to daily, oral vitamin B_{12} supplementation over periods of five to eight months. Chow *et al.*[3] suggested that the rise in serum levels reflected tissue repletion. It is unlikely that complete tissue repletion in the gilts occurred within the two weeks of oral dosage.

SUMMARY

A relationship between daily oral intake of vitamin B_{12} and total blood serum vitamin B_{12} levels was reported for nonpregnant female swine, and the significance was discussed.

A practical diet for experimental use, low in vitamin B_{12} but adequate in other nutrients according to current concepts, was described.

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Preliminary Studies on the Histopathology of Experimental Ornithosis in Turkeys

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SINCE THE DISCOVERY of the true nature of psittacosis, nearly 30 years ago, this disease has been studied in a variety of birds and mammals. The disease is usually termed ornithosis in species other than psittacine birds. Although human beings, laboratory animals, and some 70 species of birds are known to be susceptible to psittacosis-ornithosis infection, detailed histopathological studies are recorded only in the parrot,[13,21] parakeet,[13] pigeon,[10,23] mouse,[17,20] monkey,[18,20] rabbit,[19,20] guinea pig,[19,20] and man.[1,12]

The works of Irons *et al.*,[11] incriminating the turkey as a source of human infection, the isolation of ornithosis agents from turkeys in several localities,[3,4,8,14] and the public health menace in poultry processing plants [2] have stimulated research on strains of the agent isolated from turkeys. Most of the reported work with these strains has been of epidemiological, serological, or therapeutic nature with emphasis on diagnosis and control.[7] The gross lesions produced by these agents in turkeys have been reported by several workers [5,6,15,16] and the microscopic lesions of a small group of turkeys have been described by Pate.[15]

This study was undertaken to obtain further information on the pathogenesis and histopathology of these infections in turkeys.

MATERIALS AND METHODS

Tissues were obtained from the turkeys used by Davis and Delaplane to study the gross lesions of turkey ornithosis. Since the results of their study are published separately [6] and contain full details of the experimental design, these details will be omitted.

A total of 500 turkeys, 1 to 24 weeks old, were inoculated intratracheally with the Jo strain of the ornithosis agent. Portions of lung, heart, liver, spleen, and kidney from each bird and the tracheas and gonads from most turkeys were fixed in 10 per cent formalin. Tissues from another small group of turkeys were fixed in Zenker's solution because of the erratic results obtained with Giemsa's stain on formalin-fixed specimens. After paraffin embedding, tissue sections were cut at 6 μ and stained with Mayer's or Harris' hematoxylin and eosin. Selected tissues were also stained by the Gridley fungus, Gram, Prussian blue, and Gridley reticulum methods. Wolbach's modification of Giemsa's stain was used routinely on the Zenker-fixed tissue to study the distribution of elementary bodies.

RESULTS

The incidence of microscopic lesions observed in this study is tabulated (table 1).

TABLE 1—Incidence of Microscopic Lesions in Experimental Ornithosis in Turkeys

Lesion	Total No. organs examined*	Mild† (%)	Severe (%)	Total (%)
Tracheitis	276	31.0	45.0	76.0
Pneumonitis	485	19.0	36.0	55.0
Pericarditis	493	25.0	69.0	94.0
Myocarditis	493	37.0	30.0	67.0
Hepatitis	477	49.0	41.0	90.0
Splenitis	483	25.0	64.0	89.0
Orchitis	131	20.0	10.0	30.0

* Although 500 turkeys were inoculated, organs from some were not available for histopathological study.

† Lesions similar in type but not as extensive or marked as those designated severe.

Respiratory System.—Varying degrees of tracheitis were present in the majority (76%) of the infected birds. The severely affected tracheas had small amounts of mucus and a few heterophils in the lumen, and extensive infiltration of mononuclear cells, lymphocytes, and heterophils in the lamina propria and submucosa. Some mucous glands were filled with cellular debris; others were almost obliterated by the cellu-

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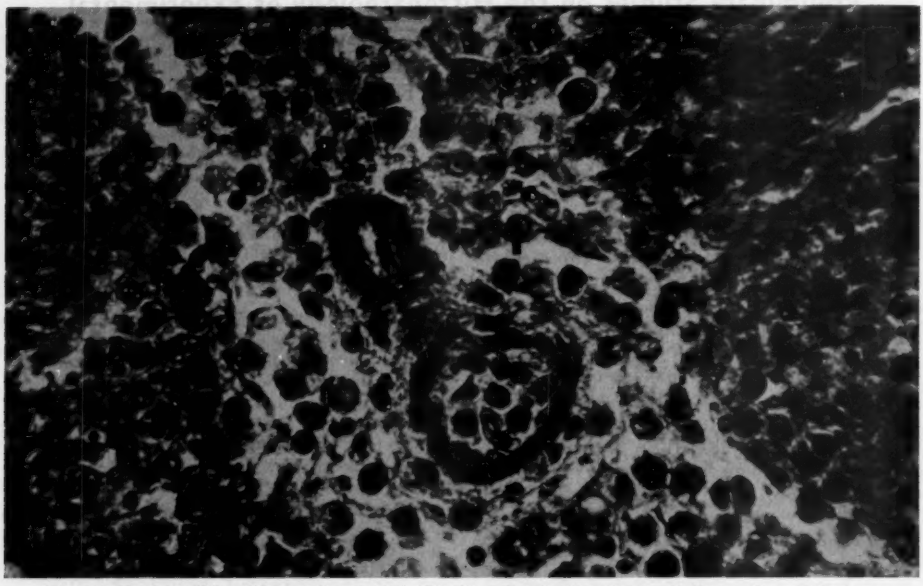

Fig. 1—Pneumonitis. Mononuclear cell infiltration (1) in the stroma between the respiratory tubules (2) of two tertiary bronchi. H & E; x 570.

Fig. 2—Pneumonitis. Cellular exudate in the lumen of tertiary bronchus (1) and the atrium (2) of the respiratory tubules. Giemsa's stain; x 380.

lar infiltration. The cilia, normally present on the respiratory epithelium, were absent in severely damaged areas.

In more than half (55%) of the infected turkeys, there was some degree of epithelioid pneumonia (pneumonitis). However, this lesion was present consistently only in turkeys less than 10 weeks old (80 to 100%), and the incidence dropped sharply as the birds matured (10 to 20%).

The pneumonitis in the severely affected birds originated at the primary bronchus and extended ventrally to involve most of that portion of the lung. An infiltration of large mononuclear cells in the normally inconspicuous stroma separated the tertiary bronchi and encroached on the respiratory tubules (fig. 1). The lumina of these tubules were obliterated by swelling of the congested capillaries, mononuclear cell infiltration, and septal cell proliferation. Varying amounts of fibrin and mononuclear cells, which apparently gained entrance through the apertures of the respiratory tubules (fig. 2), filled the lumina of the secondary and tertiary bronchi. Some mononuclear cells were relatively small with oval, deep-staining nuclei and a moderate amount of cytoplasm; others were epithelioid in appearance and possessed abundant

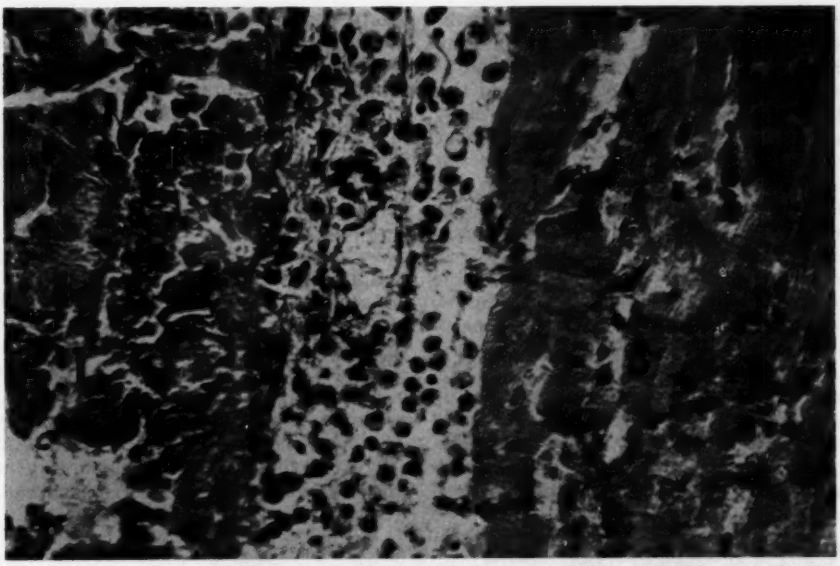

Fig. 3—Pericarditis. Epicardium thickened by inflammatory exudate consisting of fibrin (1) and mononuclear cells (2). Myocardium (3). H & E; x 400.

foamy or vacuolated cytoplasm. Lymphocytes, heterophils, and erythrocytes also were present but were never as numerous as the previously described cells.

The affected portions of the lungs were congested but, while some were edematous, marked exudation of fluid was not a prominent feature of the inflammatory process. There was necrosis of individual cells and of large areas of tissue; the parenchyma and the stroma were equally affected. In the older turkeys which were killed two weeks after inoculation, the lungs were diffusely infiltrated with lymphocytes and heterophils.

In approximately 10 per cent of the birds, the lumina of the secondary and tertiary bronchi contained eosinophilic masses of necrotic cells and fragmented nuclei. There was desquamation of the epithelia and proliferative changes in the underlying tissues of these bronchi. In approximately half of the birds, multinucleated giant cells were present at the junctions of the pulmonary tissues and the necrotic masses in the lumina. No fungi were demonstrated in any of these lesions.

In a majority of the infected turkeys, the air sacs had a fibrinous to fibrinopurulent inflammatory exudate on the respiratory and peritoneal surfaces. Numerous heterophils were in this exudate, and mononuclear cells and lymphocytes distended the intercellular spaces in the membrane proper. Differentiation of the epithelial and mesothelial surfaces of the air sacs was often difficult and sometimes impossible because of deciliation, flattening, and des-

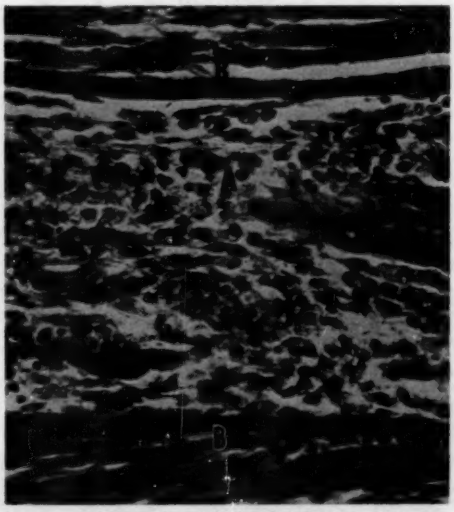

Fig. 4—Myocarditis. Cellular infiltration (A) separating the muscle fibers (B). H & E; x 333.

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quamation of the respiratory epithelium. In some birds, cystic spaces were present on the respiratory sides of the membranes, apparently produced by hyperplasia of the epithelia. Granulomas, consisting of caseation necrosis surrounded by multinucleated giant cells, were also present on the respiratory side of the membrane. While these granulomas resembled aspergillosis, mycelia were not demonstrated by fungus stains.

Vascular System.—Fibrinous to fibrinopurulent pericarditis was observed in 94 per cent of the infected birds. The pericardium and epicardium were thickened by the swelling of congested vessels and an inflammatory exudate containing fibrin, large mononuclear cells, and varying numbers of lymphocytes and heterophils (fig. 3). The fibrin occurred in the thickened membranes and on the epicardial surface where heterophils were often present. In the more mature turkeys, increased numbers of fibroblasts appeared in the epicardium and the fibrin was partially organized.

Infectious myocarditis was present in more than half (67%) of the infected birds and occurred both deep in the myocardium (fig. 4) and superficially, where it appeared to be an extension of the pericarditis. The characteristic cell was the large mononuclear macrophage with or without a few lymphocytes and heterophils.

Arteritis, involving the smaller arteries of the liver, spleen, kidney, lung, and testicle, was present in approximately 8 per cent of the infected turkeys. While this lesion was most commonly observed in the testes, there seemed to be no consistency to its occurrence in any one organ or in all organs of those birds in which the lesion was present. Usually, the arteritis was ob-

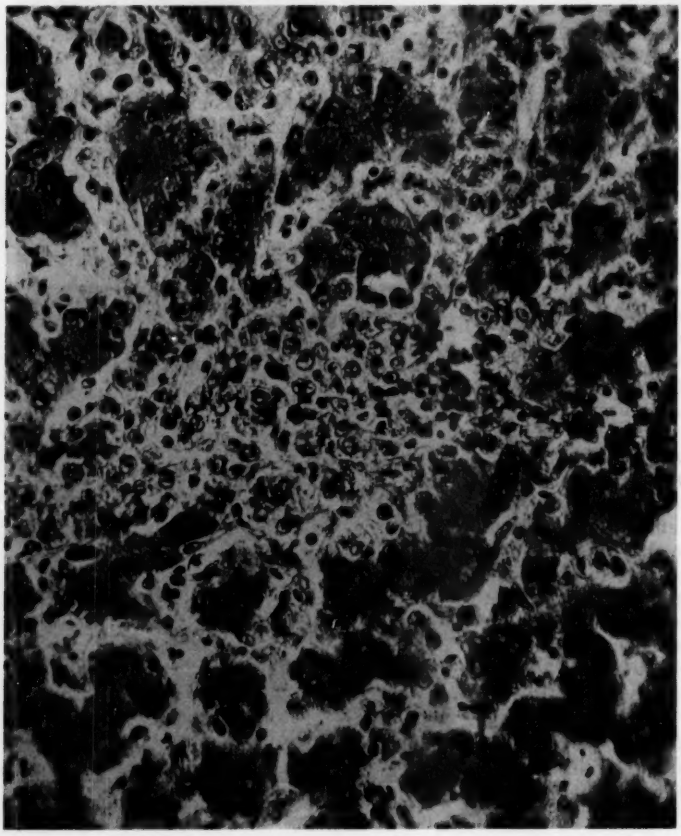

Fig. 5—Hepatitis. Focal accumulation of mononuclear cells and lymphocytes in the liver. The sinusoids contain cells of similar type. H & E; x 267.

served only in the severely damaged areas, and was characterized by hyalin degeneration of the media with infiltration of lymphocytes and heterophils. Endothelial swelling and proliferation were present in the damaged arteries and in smaller vessels which otherwise remained unchanged.

Digestive System.—Infectious hepatitis of varying intensity was present in 90 per cent of the infected turkeys. In the younger birds and the more susceptible older ones dying from ornithosis, there was diffuse dilation of the sinusoids with infiltration of mononuclear cells, lymphocytes, and heterophils. The proliferated and swollen Kupffer's cells were filled with debris and an iron positive, yellowish pigment which was considered to be hemosiderin. Necrotic hepatic cells were scattered through the organ with focal necrosis occurring rarely. As the birds became older and more resistant to the agent, the diffuse character of the infiltration gave way to one of focal nature (fig. 5). These foci were composed of the same types of cells, had no preferential location of the lobule, and occasionally contained a few necrotic cells. In mature turkeys, the cellular infiltrations were usually located only in the periportal areas and adjacent to blood vessels.

A layer of fibrin, often containing inflammatory cells, was present on the hepatic capsule of those turkeys which had gross lesions of peritonitis. Organization of the fibrin was commonly observed but its slight attachment was attested by the ease with which the exudate slipped from the organ in preparing the tissue for sectioning.

Nearly all of the livers were markedly green at necropsy, a condition considered to represent retention of bile; however, microscopic examination revealed inspissated bile in only 3 birds. Pigment, still in a dissolved state, may have diffused into the fixing solutions.

Except for a catarrhal enteritis in the acutely sick turkeys, other organs of the digestive system were without notable lesions.

Hemolytopoietic System.—The spleens of 89 per cent of the infected turkeys were altered to some extent by the infection. Reticuloendothelial proliferation (hypertrophy of the adenoid sheath) was marked in the younger turkeys with gradually decreasing severity as the birds matured (fig. 6). The proliferated cells, forming broad cuffs around the arterioles, were pale staining, often without nuclei, and many possessed vacuolated cytoplasm. Unmistakable necrosis of these areas occurred in the more severely damaged organs. These changes explained the enlarged and mottled spleens observed grossly at necropsy of infected turkeys. In some birds, the reticuloendothelial proliferation was at the expense of the red pulp, while in others there was lymphoid atrophy. Lymphoid

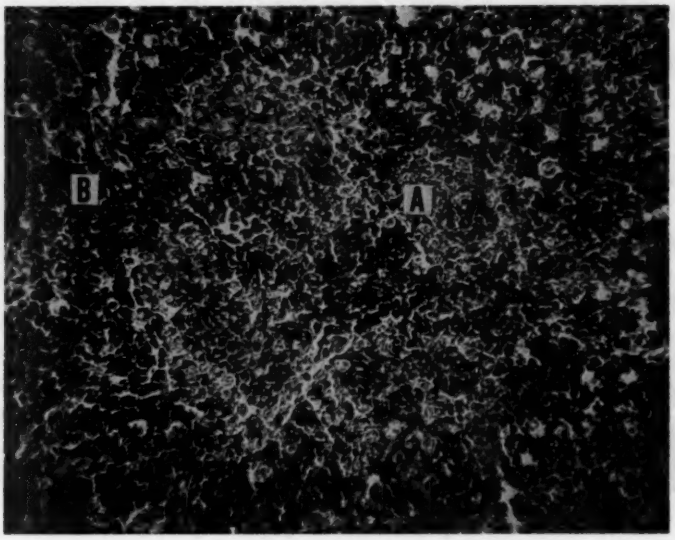

Fig. 6—Spleen. Several areas of reticuloendothelial proliferation (A) surrounding arterioles, numerous lymphocytes (B). H & E; x250.

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proliferation in both follicular and diffuse forms was present in many turkeys. There was congestion and heterophilic infiltration of the red pulp, and severe blood destruction was evidenced by numerous hemosiderin-laden macrophages. The splenic capsule was thickened and covered by fibrin, mononuclear cells, and heterophils.

Urinary System.—Degenerative changes and cellular infiltrations were present in the kidneys of 44 and 60 per cent, respectively, of the infected turkeys. However, changes identical in type but of a less severe nature were found in approximately the same percentage of the uninoculated control birds. In the young turkeys, these changes were primarily degenerative and characterized by necrosis of the epithelium of the convoluted and collecting tubules. Affected cells were swollen, often separated from the basement membrane, and possessed acidophilic or fragmented cytoplasm with pyknotic or karyorrhectic nuclei. The affected areas varied in size from small foci in a single tubule to broad areas involving many tubules. As the birds matured, lymphocytes and heterophils appeared in structureless masses in the stroma and infiltrated the areas of damaged tubules. The lamina propria of the large collecting tubules and ureters was infiltrated by similar cells.

Reproductive System.—An infectious orchitis and epididymitis was present in approximately one third (30%) of the male turkeys. In the younger birds, the changes consisted only of infiltration of lymphocytes and heterophils in the stroma. The ornithosis agent seemed to have an affinity for the active germinal epithelium, and severe orchitis occurred in the mature birds. Fibrin and inflammatory cells appeared in association with the desquamated and necrotic epithelium, filling the seminiferous tubules with an eosinophilic exudate (fig. 7). The exudate in the tubules first affected was transformed into a deeply acidophilic mass of almost hyalin consistency and occasionally contained small granulomatous nodules. The interstitial tissue was heavily infiltrated by lymphocytes and varying numbers of heterophils

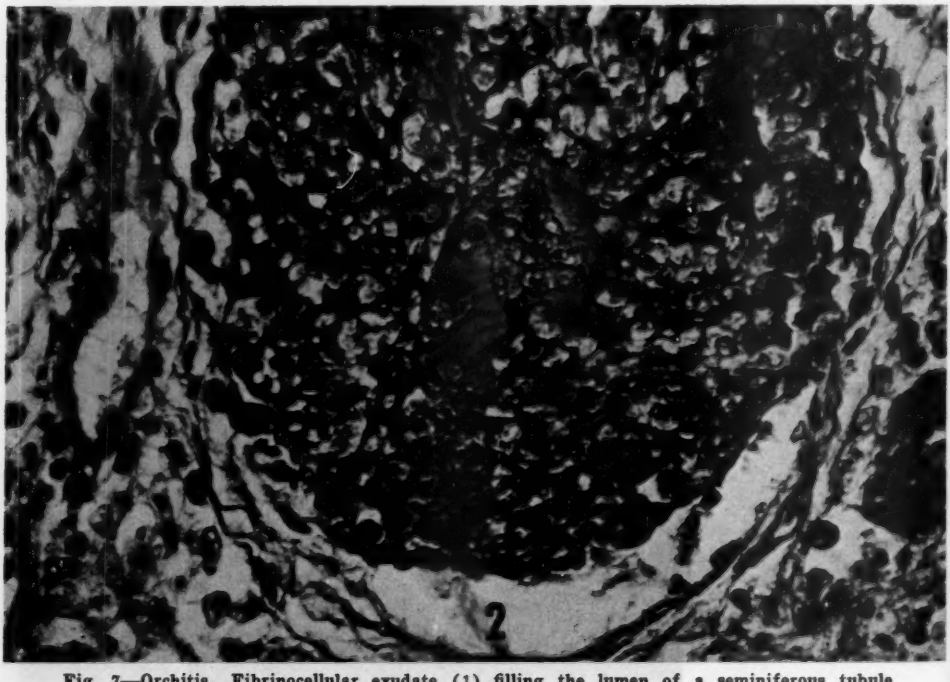

Fig. 7—Orchitis. Fibrinocellular exudate (1) filling the lumen of a seminiferous tubule. Desquamation of the germinal epithelium (2). H & E; x 500.

and monoluclear cells. These infiltrations were usually of a diffuse nature, but focal and perivascular formations were observed. The lesions of the epididymis were similar to those of the testis.

Except for changes related to extension of the inflammatory process in the associated air sacs, the ovaries presented no distinct lesions.

Nervous System.—Brains from only 6 turkeys were available for examination, and they were without significant changes.

Giemsa-Stained Tissue.—The tissues of infected turkeys, when fixed in Zenker's solution and stained by Wolback's modification of Giemsa's stain, contained small oval bodies occasionally occurring free in the inflammatory exudate but more commonly arranged in clusters in the cytoplasm of macrophages. The individual bodies varied in diameter from approximately 0.1 to 1.0 μ. Clusters composed of numerous bodies tended to consist of the minute forms, while the bodies of larger size occurred singly or in clusters composed of only a few bodies. Those of the larger size stained blue, and the smaller forms reddish-blue to purple. These bodies were not observed in the tissues of the uninfected controls and were considered to be elementary bodies of the ornithosis agent.

Exudative macrophages in the lungs (fig. 8), pericardium (fig. 9), air sacs, and trachea, the Kupffer's cells of the liver, and the reticuloendothelial cells of the spleen contained elementary bodies with varying frequency. Generally, they were most numerous in young turkeys dying from the infection and were located with greatest ease in areas in which there were necrotic cells. The germinal epithelium of the seminiferous tubules in sexually mature birds also contained elementary bodies.

Small bodies were present in the lumina of the renal tubules and in vacuolated cells of their lining epithelium. These structures appeared to be suspended in a fine reticular latticework and at times several were clumped together forming irregular masses. In hematoxylin and eosin preparations, the bodies stained faintly acidophilic. Similar bodies were also present in the kidneys of the uninfected controls and presumably consisted of urate granules.[26] Their size and staining characteristics were compatible with those of elementary bodies, from which they must be differentiated.

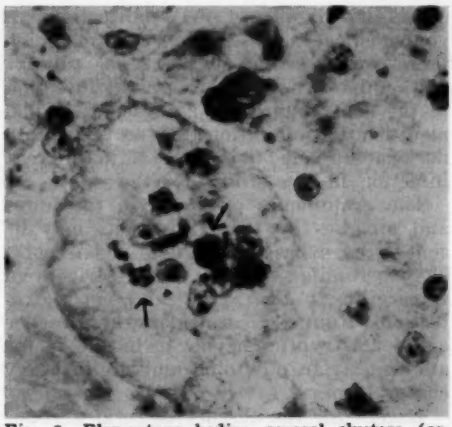

Fig. 8—Elementary bodies, several clusters (arrows), in cells of the exudate filling the lumen of a tertiary bronchus. Giemsa's stain; x 960.

DISCUSSION

The renal lesions observed in both the infected and control turkeys are difficult to explain, since no other lesion was common to both groups of birds. Pate [15] described similar lesions in infected and control birds in his study of the histopathology of turkey ornithosis. Gross,[9] working with combination *Escherichia coli* and pleuropneu-

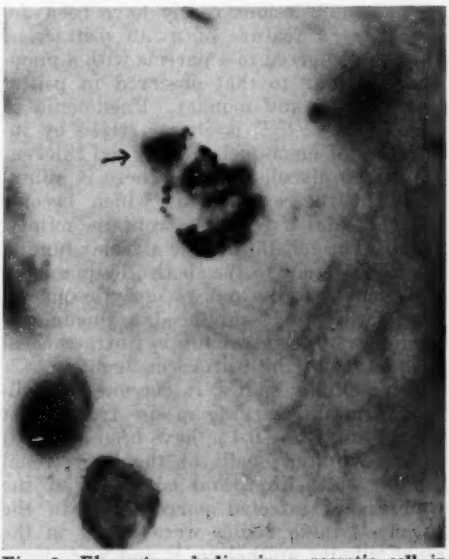

Fig. 9—Elementary bodies in a necrotic cell in the epicardium. The pyknotic nucleus (arrow) is slightly out of focus. Giemsa's stain; x 2,400.

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monia-like organism (PPLO) infections in young chickens, observed a case in which there was necrosis of the renal tubular epithelium. The lack of signs of infection in the control birds and negative complement-fixation inhibition tests on the supply flock should rule out accidental, inapparent infection in this study.

The granulomas produced in the lungs and air sacs by these infections may be considered a basic pattern of avian pathology. Similar lesions are described in Aspergillus,[24] *Esch. coli*,[9] and PPLO [27] infections. Runnells [22] considers the presence of an antitryptic enzyme to be the factor allowing avian pus to accumulate in a dry cheesy form.

Except for the absence of focal necrosis of the liver, which has been prominent in parrots [13,21] and mice [17,20] infected with psittacosis, the lesions produced in this group of turkeys by the Jo strain of the ornithosis agent are comparable with those of psittacoid agents in other species. Species differences in lesions seem to consist primarily of variations in distribution, extent, and severity.[10,13,17,20,21,24] The psittacosis-ornithosis agents produce both necrotizing and proliferative changes in the tissues of their hosts, and their inflammatory exudates are characterized by a predominance of large mononuclear cells.

Pneumonic lesions rarely have been described as a feature of avian psittacosis. Sprunt [25] referred to 4 parrots with a pneumonia similar to that observed in psittacosis of man and monkey. Pneumonia in these species [1,12,18] is characterized by infiltration of mononuclear cells and thickening of the alveolar walls. There is stimulation of the septal cells which become cuboidal and frequently contain mitotic figures. The cells filling the alveolar lumina are considered to be both desquamated "epithelial" cells and phagocytic macrophages. Rivers [18] produced a pneumonia of similar type in rabbits by intratracheal inoculation of the psittacosis agent.

Intracellular bodies, in coccoid or bacillary form and varying in size from "just visible" to 0.2 by 0.4 μ, have been observed in the Kupffer's cells of the liver, renal tubular epithelium, and islet cells of the pancreas of infected parrots.[13,21] In the parakeet, these bodies were found in the renal tubular epithelium [13] and were present in the spleen and liver of mice following intraperitoneal inoculation.[17] Smears from the pericardial and peritoneal surfaces [10] and sections of liver, spleen, and pancreas [23] from infected pigeons revealed cells containing elementary bodies.

SUMMARY

The lesions observed in 500 turkeys infected with the Jo strain of the ornithosis agent consisted of epitheloid pneumonitis, tracheitis, hepatitis, myocarditis, splenitis, and orchitis, and fibrinous to fibrinopurulent inflammation of the air sacs and serous membranes. The changes in the parenchymatous organs were both proliferative and necrotizing, the latter occurring most commonly in the younger turkeys. The large mononuclear macrophage was the most common cell type in the inflammatory exudates. Hemosiderosis of the spleen and liver gave evidence of severe blood destruction. Elementary bodies, varying in size from approximately 0.1 to 1.0 μ and occurring in clusters, were observed in exudative macrophages, reticuloendothelial cells of the spleen, Kupffer's cells of the liver, and germinal epithelium of the seminiferous tubules. Since similar changes were present in the kidneys of both the experimentally infected turkeys and the uninoculated controls, the significance of the renal lesions and their possible relationship to the ornithosis agent is unknown.

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Susceptibility of Certain Strains of White Leghorn Chickens to *Salmonella Pullorum*

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THE GENETIC NATURE of the resistance of chickens to *Salmonella pullorum* was shown by Roberts and Card.[5] Support for that observation was provided by breeding resistant progeny with the original strain to obtain progeny of greater resistance.[6] Hutt and Scholes [2] reported that the white dominant gene of the White Leghorn chicken exerted little influence on resistance to disease, as no significant difference was found in susceptibility to *S. pullorum* by the Black Leghorn and the White Leghorn birds.

For a number of years, we have employed White Leghorn chickens of the Babcock strain in our investigations of the host-parasite relationship.[1,7] The $l.d._{50}$ for this strain has always been low, in the order of 1,000 cells. Recently, however, we used other strains of the White Leghorn chicken with variable results.

In view of evidences of a variation in susceptibility to *S. pullorum* between breeds, and the conferring of resistance through selective breeding, it appeared that an investigation of the susceptibility of different strains of White Leghorn chickens would be desirable. It was important because of difficulties encountered by us in establishing the $l.d._{50}$ for *S. pullorum* in chicks of that breed obtained from commercial hatcheries without particular reference to strain.

MATERIALS AND METHODS

White Leghorn chickens of five different strains were used in the present investigation of susceptibility to *S. pullorum*. The principal test organism was *S. pullorum* CDC 3522/51 which had been used in all our previous host-parasite studies because of its ability to maintain a stabilized degree of virulence. A second strain of *S. pullorum* (CDC 533) was also used in this study for comparative purposes.

The cell suspensions were prepared by suspending the growth from 22 to 26-hour trypticase soy agar (B.B.L.) slants in saline solution and adjusting the density to give a reading of 125 in the Klett-Summerson photocolorimeter.* The inoculums were prepared by making tenfold dilutions from the adjusted suspension.

TABLE 1—Logarithm of the $l.d._{50}$ of Salmonella Pullorum for Rhode Island Red Chickens and for White Leghorn Chickens of the Babcock and West Line Strains

Bacterial strain	Rhode Island Red	White Leghorn	
		Babcock	West Line
CDC 3522/51	1.05	3.05 ± 0.66	4.89 ± 0.35
CDC 533		2.21 ± 0.04	3.88 ± 0.19

Chicks, 24 hours old, were placed in groups of eight and inoculated intraperitoneally with 1 ml. of the desired dilution. Deaths were recorded over a period of ten days and the number of deaths at that time was used in the calculation of the $l.d._{50}$ according to the method of Reed and Muench.[4]

RESULTS

The relative susceptibility of five commercial strains of White Leghorn chickens was determined by inoculation with $10^{2.9}$ cells. The order of increasing susceptibility, based on the number of survivors ten days after inoculation, was West Line, Mount Hope, Ghostly, Strain Cross, and Babcock strains. Accordingly, the Babcock and the West Line strains were chosen for further studies, as they were considered to represent the extremes of susceptibility to *S. pullorum*-CDC 3522/51. As a means of relating this work to previous observations, the $l.d._{50}$ for Rhode Island Red cockerels also was determined.

The differences in the $l.d._{50}$ for the Babcock and West Line strains showed the former to be much more susceptible to in-

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* The Klett-Summerson photocolorimeter is manufactured by the Klett Manufacturing Co., New York, N.Y.

fection with the two strains of *S. pullorum* than the latter (table 1). The $l.d._{50}$ for the Rhode Island Red chickens infected with *S. pullorum* CDC 3522/51 was $10^{1.05}$.

Discussion

The difference in breed susceptibility to *S. pullorum* as originally reported by Hutt and Scholes [2] has been confirmed. The $l.d._{50}$ for the Babcock strain of White Leghorn chickens was about 100 times that of the Rhode Island Red, while that of the other White Leghorn strains tested was even greater.

A marked difference has been found to exist between the susceptibilities of two of the test strains, the Babcock and West Line. As stated previously, Roberts *et al.*[6] found special resistant strains could be bred by crossing the resistant (survivors of experimental infection) birds with those from the control flock. These workers attributed the differences in susceptibility to numbers of lymphocytes and reported increases in lymphocytic cells by the resistant strain of the bird. More recently, it has been demonstrated that the differences in resistance of White Leghorns and heavy breeds to pullorum disease may be due to the higher body temperature of the former.[3, 8]

Neither the average body temperature nor the leukocyte count were included as a part of this study. Therefore, no conclusion can be drawn concerning the reasons for the difference in strain susceptibility, but the contributing factors may be the same as between breeds.

The susceptibility of the chickens, on the basis of the $l.d._{50}$, is in accord with the observations cited, except that the existence of differences in susceptibility by commercial strains has not been previously reported.

Summary

Evidence was obtained to show differences in breed susceptibility to pullorum disease. A difference in the $l.d._{50}$ of two strains of *Salmonella pullorum* for each of two strains of White Leghorn chickens was observed.

While previously reported causes of susceptibility or resistance to infection with *S. pullorum* were not investigated, it has been suggested that they may account for differences between strains as well as breeds.

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Factors Affecting Interference with Newcastle Disease Infection

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NUMEROUS WORKERS who have demonstrated interference between both homologous and heterologous active viruses have been listed by Henle.[11] Infectious bronchitis virus (IBV) can interfere with Newcastle disease virus (NDV) multiplication in embryonating eggs[18] and in young chickens,[7, 9, 13] when both viruses are administered simultaneously. In those studies, IBV had been given an advantage of time or dosage. Markham *et al.*[19] reported that they were unable to demonstrate interference of NDV by IBV when both viruses were mixed in high concentrations and given simultaneously or when IBV was given in advance of NDV. Hanson *et al.*[9] failed to demonstrate interference of NDV when IBV was given seven days in advance.

Some workers [1, 12, 22, 23] have observed interference of active viruses by inactive homologous viruses. Interference under these conditions is produced by giving the inactive virus the advantage of an excessive dose, which usually is administered in advance of the active agent. Bang[2] observed interference of active NDV in chicken embryos by prior exposure to heat-inactivated NDV. Groupé[6] demonstrated that, in chicken embryos, heat-inactivated IBV interfered with the multiplication of active IBV which was inoculated later.

Recently, Isaacs and Lindenmann[15] and Isaacs *et al.*[16] reported that a substance, "interferon," produced when heat-inactivated influenza virus was incubated at 37 C. in the presence of normal chorioallantoic membranes, interfered with the growth of several viruses. It inhibited the growth of homologous influenza viruses, NDV, vaccinia, and some myxoviruses in tissue culture studies. Interferon was destroyed at 60 C. but survived 56 C. for one hour.

Some virus-free substances also have been shown capable of interfering with viral activities. Hardy and Horsfall[10] found that normal allantoic fluid contained a component which interfered with the hemagglutination ability of influenza virus.

Mammalian serums also contain a substance which is capable of inactivating viruses. Howitt,[14] Bang *et al.*,[3] and Ginsberg and Horsfall[5] demonstrated that fresh mammalian serums contained a heat-labile substance which inactivated NDV. Karzon[17] found an inhibitor substance, present in the tissues of normal animals, that neutralized the viral-inhibiting substance in normal animal serums. Other workers[20] suggested that the properdin system (properdin, complement, and magnesium ions) is responsible for this reaction.

The purpose of this paper is to reconfirm earlier studies of IBV interference with NDV and to report investigations of the interfering effect of heat-treated IBV on virulent NDV in chickens. Normal horse serum and normal allantoamniotic (AA) fluid were tested also for interfering effects. Virus sensitization of chickens was also attempted to reconfirm previous NDV studies of Hanson *et al.*[8]

MATERIALS AND METHODS

The chickens used in these experiments were White Plymouth Rocks, 4 to 8 weeks old. Composite serum samples were obtained by taking blood samples and pooling equal volumes of serum from 5 chickens of each group before exposure to the viruses. The serum samples were tested for Newcastle disease and infectious bronchitis antibodies. Newcastle disease antibodies were detected with the *alpha* hemagglutination-inhibition test, and a serum-neutralization (SN) test was used to detect infectious bronchitis antibodies. Birds were divided at random into groups; each group was placed in isolation quarters prior to exposure and was attended by a separate caretaker.

The viruses used throughout these experiments were the California strain 11914 of NDV and the Illinois strain 70768 of IBV. The viruses were propagated in 9- to 11-day embryonating chicken eggs at 37 C. for 36 to 48 hours and cooled to 4 C. three to six hours before harvesting. The virus-infected AA fluids were pooled from both living and dead embryos; they were held at −20 C. until used. Nutrient broth was used to dilute NDV for exposure of chickens so that each 0.1 ml. contained approximately $10^{4.0}$ 50 per cent embryo lethal doses ($e.l.d._{50}$). For dual exposure, the NDV was diluted and then combined with an equal portion of IBV approximately one hour before inoculation. Ap-

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TABLE 1—Effects of Heated and Unheated Infectious Bronchitis Virus on a Subsequent Newcastle Disease Infection

Inoculum	Route	No. chickens	Mortality	c.n.s. disturbance	Total loss (%)	*Alpha* HI positive
IBV (56 C. 15 min.)	i.v.					
NDV (15 min. later)	i.n.	14	2	1	21.4	7/11
IBV (untreated)						
NDV (simultaneously)	i.n.	15	0	0	0.0	0/15
NDV	i.n.	15	5	5	66.0	5/5
IBV (untreated)	i.n.	15	0	0	0.0	0/15

IBV = infectious bronchitis virus; NDV = Newcastle disease virus; i.v. = intravenous; i.n. = intranasal; c.n.s. = central nervous system disturbance at 14 days.

proximately $10^{4.5}$ 50 per cent embryo infective doses ($e.i.d._{50}$) of IBV in undiluted AA fluid was used in all cases.

The IBV was treated in a water bath at 56 C. for 15 minutes in experiment 1 and for two hours in experiment 2. The IBV was heated first for 15 minutes, as available information indicated this was an adequate interval;[4] however, a two-hour period was necessary to completely inactivate the virus in experiment 2. The normal AA fluid was obtained from 10- to 12-day embryonating chicken eggs and was heated for various intervals. The normal horse serum was heated at 56 C. for one hour. Control groups of chickens received heat-treated IBV, AA fluid, and horse serum in the corresponding experiments.

All inoculations involving NDV and untreated IBV were made with drops, intranasally, each chicken receiving approximately 0.05 ml. of the virus. Inoculations of 3 to 5 ml. of heat-treated IBV, normal AA fluid, and normal horse serum were made intravenously in wing veins. The intravenous route was chosen so that the heat-treated virus would have the advantage of time and a large volume. Intravenous inoculations were made 15 to 30 minutes prior to the intranasal exposure with NDV except in experiment 3, when one group received AA fluid 24 hours in advance of NDV. Chickens that were dead or showed incoordination at the end of the 14-day observation period were listed as losses.

RESULTS

Experiment 1.—This experiment was planned to determine whether heat-inactivated IBV would interfere with NDV. The IBV heated at 56 C. for 15 minutes produced some precipitate which caused anaphylactic shock when injected intravenously in chickens. After this observation, the precipitate was allowed to settle, and only the supernatant fluid was injected into the chickens. Each chicken in group 1 was given 5 ml. of heat-treated IBV a few minutes before intranasal exposure with NDV. Chickens in group 2 were given both untreated IBV and NDV, intranasally. The chickens in group 3 were given NDV, and birds in group 4 were given untreated IBV. These chickens were observed daily after inoculation, and the results of a subsequent 14-day observation period are summarized (table 1). The loss in group 1, where chickens were given the heat-treated IBV prior to NDV exposure, was 21.4 per cent, as compared with 66.0 per cent of the NDV control group. There were no deaths among chickens given untreated IBV and NDV simultaneously. These results suggest that untreated IBV, when given intranasally along with NDV, and heat-treated IBV, when given intravenously prior to exposure to NDV, had an inhibitory effect on Newcastle disease infection.

Experiment 2.—This experiment was planned to investigate further the interfering effect of heat-treated IBV and also to test the effect of heat-treated normal AA fluid. The IBV was heated at 56 C. for two hours and the normal AA fluid was held at 56 C. for one hour.

TABLE 2—Effect of Heated Infectious Bronchitis Virus, Normal Allantoamniotic Fluid, and Unheated Infectious Bronchitis Virus on a Subsequent Newcastle Disease Infection

Group inoculum	Route	No. chickens	Mortality	c.n.s. disturbance	Total loss (%)	*Alpha* HI positive
1. IBV (56 C. 2 hr.)	i.v.	20	8	1	45	10/11
NDV (30 min. later)	i.n.					
2. AA (56 C. 1 hr.)	i.v.	13	2	1	23	7/10
NDV (30 min. later)	i.n.					
3. IBV (untreated)	i.n.	20	0	0	0	2/20
NDV (simultaneously)						
4. NDV	i.n.	20	9	0	45	5/5
5. IBV (untreated)	i.n.	20	0	0	0	0/20

IBV = infection bronchitis virus; NDV = Newcastle disease virus; AA = allantoamniotic fluid; i.v. = intravenous; i.n. = intranasal; c.n.s. = central nervous system disturbances at 14 days.

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Birds in group 1 were given 4 ml. of heated IBV intravenously, followed by NDV given intranasally. In group 2, each chicken was given 4 ml. of heated, normal AA fluid, followed by NDV intranasally. The chickens in group 3 were simultaneously given untreated IBV and NDV intranasally. Only NDV was given intranasally to chickens in group 4. Birds in group 5 were given untreated IBV. All groups contained 20 chickens except group 2, which was composed of only 13 birds.

The results of this experiment are summarized (table 2). They show that the IBV heated at 56 C. for two hours failed to exert an inhibitory effect as compared to birds given NDV only. However, heat-treated normal AA fluid did reduce the mortality from NDV infection. The chickens given the mixture of untreated IBV and NDV had no losses; however, the serum of 2 birds had *alpha* hemagglutination-inhibition titers of 1:80 and 1:640.

Ten of the chickens with negative hemagglutination-inhibition serum titers from group 3 and 10 normal chickens of the same age which had been held as normal controls were inoculated two weeks after the previous inoculation period to ascertain whether the sensitivity of the former to NDV had been altered. The twice-exposed chickens suffered an 80 per cent death loss as compared to a 40 per cent death loss among the previously unexposed control chickens. This result is similar to those obtained previously by us, in which it was found that chickens exposed to small quantities of NDV may be more susceptible to the same virus on re-exposure.[9]

Experiment 3.—Heat-treated normal AA fluid and normal horse serum were each tested for possible inhibitory effects on NDV infections in chickens. In addition, AA fluid was given simultaneously and 24 hours prior to NDV administration to determine whether time might be a factor. The normal AA fluid was heated to 56 C. for 15 minutes. Allantoamniotic fluid was given to 25 chickens in group 2 just prior to exposure with NDV. The chickens in group 1 were given 4 ml. of AA fluid 24 hours before intranasal exposure to NDV. Three milliliters of horse serum which had been filtered through a Seitz EK filter and heated at 56 C. for one hour was administered to each of 20 chickens in group 3 just prior to intranasal exposure with NDV. Group 4 consisted of 25 chickens exposed to NDV alone. Each chicken in groups 5 and 6 was given untreated IBV and NDV, intranasally; however, the chickens in group 5 were exposed to both viruses simultaneously, while the chickens in group 6 were given IBV 24 hours previous to exposure to NDV. The chickens in group 7 were given untreated IBV.

The results of this experiment at two weeks after its initiation are summarized (table 3). No inhibitory effect was produced at either time interval by AA fluid, heat treated 15 minutes, but horse serum exerted an inhibitory effect since losses were only 50 per cent in the principals as compared to 80 per cent in the NDV control birds. Group 5 had a 16 per cent loss and group 6 had a 24 per cent loss, indicating that untreated IBV caused interference when given 24 hours previous to as well as simultaneously with NDV.

Experiment 4.—In the preceding experiment, the AA fluid heated only 15 minutes failed to cause interference with NDV, so an extension of the heat period to one hour was used in this experiment. Heat-treated horse serum was used to determine whether the results obtained in experiment 3 could be reconfirmed. The chickens in group 1 were given 5 ml. of the heat-treated AA fluid intravenously before exposure to NDV. Filtered horse serum (4 ml.), heated at 56 C. for one hour, was given intravenously to each chicken in group 2 before intranasal exposure to NDV. The chickens in group 3 were given only NDV. There were 15 chickens in each of the three groups.

The results of this experiment are summarized (table 4). The death loss in group 1 was 25 per cent, and in group 2, 12 per cent as compared with 66 per cent in group 3, which received only NDV.

In several of the experiments in which chickens that were exposed to NDV and IBV simultaneously were observed longer than two weeks, paralysis of the central nervous system and deaths typical of Newcastle disease occurred in some of the birds. The onset of the signs in these cases was first evident between 16 and 21 days.

DISCUSSION

Untreated IBV, administered simultaneously with NDV, produced marked interference with Newcastle disease infection, as indicated by the reduction of losses (deaths and paralysis) of chickens (tables 1, 2, and 3). Similar findings had been reported by

TABLE 3—Effect of Heated Allantoamniotic Fluid, Heated Horse Serum, and Unheated Infectious Bronchitis Virus on a Subsequent Newcastle Disease Infection

Inoculum	Route	No. chickens	Mortality	c.n.s. disturbance	Total loss (%)
1. AA (56 C. 15 min.)	i.v.	25	21	3	96
NDV (24 hr. later)	i.n.				
2. AA (56 C. 15 min.)	i.v.	25	19	2	84
NDV (15 min. later)	i.n.				
3. h.s. (56 C. 1 hr.)	i.v.	20	9	1	50
NDV (15 min. later)	i.n.				
4. NDV	i.n.	25	19	1	80
5. IBV and NDV (simultaneously)	i.n.	25	2	2	16
6. IBV	i.n.	25	6	0	24
NDV (24 hr. later)	i.n.				
7. IBV	i.n.	10	0	0	0

AA = allantoamniotic fluid; h.s. = horse serum; c.n.s. = central nervous system disturbances at 14 days; i.v. = intravenous; i.n. = intranasal; IBV = influenza bronchitis virus; NDV = Newcastle disease virus.

Hanson *et al.*[9] The degree of interference varied from partial to complete reduction of losses. In experiment 3, deaths of a few chickens occurred in both groups which received IBV and NDV (table 3, groups 5 and 6). However, no deaths occurred in the corresponding group in experiment 2 (table 2, group 3), and 18 chickens were fully susceptible to Newcastle disease infection, as demonstrated by hemagglutination-inhibition tests and challenge with NDV. The period of interference was limited to 14 to 21 days in chickens which failed to develop detectable antibody response to the NDV. This suggests that IBV ($10^{4.5}$ e.i.d.$_{50}$), when given simultaneously, will inhibit the multiplication of NDV (10^{5} e.i.d.$_{50}$) in some birds to such a degree that detectable Newcastle disease antibodies are not produced.

Intranasal administration of IBV, 24 hours before exposure to NDV, interfered with the NDV infection in the chickens (table 3) similar to interference when the viruses were given simultaneously. In this group of chickens, the mortality was only 24 per cent, as compared to the 80 per cent mortality sustained by chickens given only NDV. This suggests that IBV is able to interfere with NDV infection when given orally, either simultaneously or 24 hours preceding exposure to NDV. In a previous study,[9] there was no evidence of interference of NDV infection in chickens given IBV one week before NDV exposure.

Intravenous administration of IBV, AA fluid, and horse serum heated to 56 C. for one half to one hour appeared to make chickens less susceptible to intranasal exposure to NDV. However, the reactions appeared to be nonspecific and are not explained by the mechanism of viral interference.[12] Isaacs and Lindenmann[15,16] reported that heat-inactivated influenza virus incubated with normal chorioallantoic membranes produced the nonspecific substance interferon. Although this substance was nonspecific in its interfering capacity on the growth of influenza, vaccinia, Newcastle disease, and some myxoviruses in tissue culture studies, its interfering capacity was related to the concentration of influenza virus. The mechanism of the refractory capacity set up by prior inoculation with heated, normal AA fluid and horse serum may be explained by the properdin system, as the blood of the inoculated birds may have supplied the factor or factors destroyed by heating.

The NDV sensitization observed earlier[9] was again demonstrated in experiment 2. The state of NDV sensitization was not obtained in all individuals but, under these circumstances, 80 per cent of the birds reexposed showed an increased susceptibility

TABLE 4—Interfering Effects of Heated Allantoamniotic Fluid and Horse Serum on Subsequent Newcastle Disease Infection

Inoculum	Route	No. chickens	Mortality	c.n.s. disturbance	Total loss (%)
1. AA (56 C. 1 hr.)	i.v.	15	4	0	25
NDV (30 min. later)	i.n.				
2. h.s. (56 C. 1 hr.)	i.v.	15	0	2	12
NDV (30 min. later)	i.n.				
3. NDV	i.n.	15	10	0	66

AA = allantoamniotic fluid; h.s. = horse serum; c.n.s. = central nervous system disturbances at 14 days; i.v. = intravenous; i.n. = intranasal.

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to the homologous strain of NDV. This sensitized state may result from a mixed infection or from a previous single infection as described by Verge and Placidi.[21] Viral sensitization may play a significant role in some failures of vaccination, as suggested by Hanson.[8] Chickens which had a negative hemagglutination-inhibition test status and which survived an earlier simultaneous administration of IBV and NDV were found hypersensitive to re-exposure to NDV.

SUMMARY

Simultaneous exposure of chickens to infectious bronchitis virus (IBV), strain 70768, and Newcastle disease virus (NDV), strain 11914, resulted in interference of Newcastle disease infection by IBV. The interference was evidenced by absence of signs, decreased antibody production, and reduced losses. Administration of IBV 24 hours in advance of NDV also resulted in interference of NDV infection by IBV. The period of IBV interference with NDV infection produced by dual exposure persisted for 16 to 21 days.

Intravenous administration of heat-treated, normal AA fluid and horse serum a short time before intranasal exposure to NDV decreased the susceptibility of chickens to NDV.

Increased NDV susceptibility was demonstrated two weeks after simultaneous exposure to NDV and IBV.

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Role of Adjuvants in Immunogenicity of Killed Newcastle Disease Vaccines

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THE IMPORTANT ROLE of adjuvants in enhancing the effectiveness of killed vaccines is evident from various reports concerned with the investigation of this problem. In the past, many materials have been tested in the search for an adjuvant that would assure a heightened antibody response following the injection of antigenic material into the human or the animal body. The purpose of the current study was to determine the comparative efficiency of phosphorylated hesperidin, ethylene glycol, and aluminum hydroxide as adjuvants in inactivated Newcastle disease vaccines.

REVIEW OF LITERATURE

As early as 1931, Glenney *et al.*[20] demonstrated that alum was an effective adjuvant for improving the antigenicity of diphtheria toxin. Later, Freund and Bonanto[17] investigated the combined effect of paraffin oil or lanolin-like substances and killed tubercle bacilli on the immunizing effect of diphtheria toxoid and *Bacterium typhosum*. They reported that these substances produced a greater enhancing and sustaining effect on immunity than did alum or peanut oil.

Freund *et al.*[18] also investigated the effect on antibody formation of several lipoid substances, such as paraffin oil, oleic acid, anhydrous lanolin, lecithin, and peanut oil, when combined with such emulsifiers as Falba, Arlacel A, Tween 80, and myricin combined with cholesterol. The investigators found that these substances influenced the ability of the typhoid bacillus to produce antibodies or to alter the sensitivity of guinea pigs.

Freund's lipoid adjuvants were widely investigated by various research workers. In the first experiments with man, Henle and Henle[24] obtained excellent antibody response after the injection of influenza vaccines containing mineral oil and Falba; unfortunately, nodules were frequently observed in the subcutaneous tissues, and abscess formation was occasionally noted. The occurrence of these local tissue reactions discouraged further use of emulsified oil preparations until Salk and Laurent[51] discovered that vaccines in water-in-oil emulsions could be used safely in monkeys if nonirritating reagents were injected intramuscularly, rather than subcutaneously. In experiments with man, Salk *et al.*[50] confirmed this marked enhancement and persistence of antibody response without undesirable side effects.

Later, Salk[49] reported that the destructive tissue changes observed in experimental animals inoculated with influenza vaccine containing oil and an emulsifying agent, Arlacel A, were due to impurities contained in this emulsifying agent. When the impurities were removed, no unfavorable results were observed in either laboratory animals or man.

Additional data regarding the role of oil emulsions of various antigenic systems can be found in the reports of Freund *et al.*,[19] who worked with *Plasmodium knowlesi;* Lewandowski and Martin,[33] who worked with a triple antigen consisting of alum-precipitated diphtheria, tetanus toxoid, and *Haemophilus pertussis;* Philip *et al.*,[45] who worked with influenza vaccine; Dubos *et al.*,[13] who worked with a methanol extract of the tubercle bacillus; Spencer *et al.*,[56] who worked with *Micrococcus pyogenes* toxoid and bacterin; and Cutchins and Warren,[9] who worked with toxoplasma.

Although the mechanism of the action of these adjuvants has not been fully elucidated, it is generally agreed that it is due to a prolonged absorption of antigen from the injection site. Herdegin *et al.*,[28] who investigated the fate of antigen [*Shigella paradysenteriae* type III(z)] in mineral oil emulsion at the site of subcutaneous inoculation, expressed the opinion that the deterioration of antigen at the site of inoculation was paralleled by a fall in the agglutinin titers of the mice receiving the vaccine.

Cutchins and Warren[8] combined a Freund-type adjuvant with killed toxoplasma and obtained a considerable amount of complement-fixing antibodies, which could not be obtained from the injection of similar animals with an aqueous suspension of the agent. They attributed the adjuvant action to retarded dispersion of antigenic material, irritation of tissues, and nonspecific stimulation of lymphocytic activity.

Balducci[2] presented data on the mechanism of action of oily adjuvants in which the viruses of influenza, poliomyelitis, herpes, and Newcastle dis-

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ease were suspended. The author attributed the action of lipoid adjuvants to the following factors: physical (diffusion), physicochemical (surface tension), chemical (chemotaxis), and biological (tissue irritation). Balducci referred to the site of injection as the "punto strategico" or "organo" of antibody formation.

Another adjuvant, which has been widely used in the preparation of inactivated immunizing agents, is aluminum hydroxide. Wilson and Miles [60] mentioned reports by Hektoen and Welker, Cohen and Mosko, and Holford, Ludden, and Stevens concerning the ability of this substance to adsorb protein *in vivo*, as well as a report by Farago and Ujhelyi on a satisfactory immune response in guinea pigs produced by an aluminum hydroxide adsorbed diphtheria toxoid.

After working with an aluminum hydroxide adsorbed influenza virus (PR8), Henneberg and Drescher [25] felt that the immune response was associated with the surface area of the adsorbent, the amount of virus adsorbed and injected, and the presence of substances which possessed the ability to influence the surrounding tissue. These workers observed that two or three days after injection of a vaccine containing aluminum hydroxide, about 50 per cent of the adjuvant was resorbed. When examined at later intervals during the 35-day test period, the amount of aluminum hydroxide left at the site of injection was constant. Drescher [11] reported that the degree of adsorption of the virus to the adjuvant was dependent upon two factors, molarity and pH. Drescher [12] also succeeded in developing a method for the determination of the virus content in extirpated deposits of PR8 influenza virus vaccines that had been adsorbed on *gamma* aluminum oxide.

Kolmar [34] observed that the negative phase in antibody production was diminished or completely suppressed after the injection of mice with an aluminum oxide adsorbate bacterin made with killed *Salmonella typhimurium*. Koch [33] reported a marked enhancement of immunity in chickens after injection of an aluminum hydroxide-adsorbed mumps vaccine. In the course of studies of a killed foot-and-mouth disease vaccine containing aluminum hydroxide, Gribanov [22] observed that insoluble particles contained in the vaccine improved the antigenic response significantly. These findings were not confirmed by Pyl and Heinig,[47] who worked with a similar vaccine. Reports regarding the formation of cysts, abscesses, and fatty degeneration after the use of aluminum hydroxide-adsorbed pertussis, diphtheria, and tetanus vaccines have been made by Grahlow,[21] Sauer *et al.*,[52] and Lapin.[35] No explanation or reason for the appearance of such damaging effects was given.

Another type of adjuvant was described by Fazekas de St. Groth *et al.*,[16] who worked with influenza virus and succeeded in producing an increased accumulation of antibodies in the immediate environment of infectible cells by the nasal administration of preparations unrelated to influenza, such as the virus of vaccinia, metaperiodate ion, tannic acid, or zinc sulphate, to animals at the time of vaccination by extrarespiratory routes. This effect, called pathotopic potentiation of immunity, was due to agents capable of causing such an accumulation of antibodies at the site of possible infection. The authors named them pathotopic adjuvants. Fazekas de St. Groth [15] and Fazekas de St. Groth *et al.*[16] attributed the action of these materials to stimulation of mild inflammation, action on cellular receptors, and alteration in tissue and capillary permeability.

Salts of calcium and magnesium were reported by Ramon to possess the adjuvant activity in enhancing antibody responses.[60] Salk [48] investigated the effect of calcium chloride injected with influenza virus into mice. He observed no enhancement of antibody titer when compared with that produced by injection of virus only. Histological examination revealed typical foreign body reaction with a marked reticuloendothelial response.

It was known for a long time that the absorption from a site of injection of vaccine may be reduced and depot effect produced by hyaluronidase inhibition. Preston *et al.*,[46] Moss *et al.*,[42] and Beiler and Martin [4] have shown that phosphorylated hesperidin possessed this property when they experimented with this substance *in vitro* and *in vivo*. The mechanism of action of this material was attributed by Cohen *et al.*[9] to its antihyaluronidase as well as antiproteolytic properties. Martin *et al.*[39] demonstrated an adjuvant action of phosphorylated hesperidin by the enhanced antibody response to typhoid vaccine. Moss *et al.*[44] obtained the adjuvant response of phosphorylated hesperidin with vaccines prepared from influenza PR8 virus, Columbia SK virus, and diphtheria and tetanus toxoids. The antibody response in guinea pigs, rabbits, and mice observed by the authors did not differ from that obtained with an identical virus prepared with water-in-oil emulsion, and the activity of this material was probably due to a depot-forming effect.

In explaining the action of various adjuvants, Wilson and Miles [60] expressed the view that the local tissue reaction to some adjuvants probably affects cells capable of antibody production, or that adjuvants act by increasing the number of antibody-forming centers in other parts of the body.

A considerable amount of work has been reported from various countries on the preparation of killed Newcastle disease (ND) vaccines with various adjuvants.

Acevedo and Mendoza [1] introduced potassium alum and obtained an enhancement of immunity in birds inoculated with a vaccine containing this type of adjuvant. Birds vaccinated with alum vaccine were able to withstand inoculations of 500 to 1,000 m.l.d. of virus, whereas those given vaccine without potassium alum succumbed to infection. Glycerinated phosphate buffer tested by these workers did not manifest an adjuvant activity when incorporated into a killed ND vaccine.

Doyle and Wright [10] presented evidence that ND vaccine containing ethylene glycol as an adjuvant and crystal violet as a bactericidal agent was in-

capable of transmitting infection to susceptible contact birds and conferred an immunity lasting at least 12 months, as measured by serum neutralization (SN) and challenge studies. The authors emphasized the importance of immunogenicity of strains of virus in production of this type of killed ND vaccine. Working with vaccine prepared by the method of Doyle and Wright, Legenhausen and Sinkiewicz [39] stressed the importance of revaccination in obtaining the maximum protection against challenge exposure of chickens under experimental conditions. Legenhausen *et al.*[37] also emphasized the role of interval between doses of vaccine made from ethylene glycol in enhancing the maximum immune response.

Mitchell and Walker [40] succeeded in preparing a ND vaccine using a combination of mineral oil as an adjuvant and Falba as an emulsifier. A vaccine of this type produced a considerable degree of resistance, which lasted at least six months against ND. In further trials, these investigators [41] obtained evidence that such a vaccination resulted in stimulation of immunity lasting over 13 months. Field trials conducted by Mitchell *et al.*[42] revealed that 90 per cent of birds given a vaccine containing Falba and mineral oil were solidly immune at least six months after vaccination.

At the present time, aluminum hydroxide is a commonly used adjuvant. It was used first by Traub [55] in the preparation of ND vaccine. The reported immunity obtained in chickens with this product was ten times higher than that obtained with a vaccine containing no aluminum hydroxide. Traub [56] obtained this effect after double vaccination.

Working with alumina gel-adsorbed ND vaccines, Huebner [31] pointed out that tissue maturity and sensitization of the immunity-producing mechanism were two important factors in enhancing the antibody response of chickens and turkeys vaccinated with alumina gel-adsorbed vaccine. Hanson *et al.*[25] compared the immunogenicity of five strains of Newcastle disease virus (NDV) from which five alumina gel vaccines were prepared. All vaccines afforded considerable protection to a large challenge dose of virus at both four and nine weeks after vaccination.

Hofstad [27-29] emphasized the importance of intervals between injections of alumina gel-adsorbed ND vaccines in enhancing immunity. Spalatin [53] observed a difference in the duration of immunity under field and laboratory conditions while experimenting with a similar type of adsorbate vaccine. Schäfer [53] made attempts to develop a purified, concentrated NDV adsorbed to *gamma*-aluminum hydroxide and succeeded in obtaining a highly antigenic product. Hanson *et al.*[23] suggested the use of heterologous challenge strain of NDV in studies of susceptibility and resistance to infection in chickens vaccinated with killed adjuvant-containing ND vaccines. Doll *et al.*[9] reported on the failure of alumina gel ND vaccine to provide protection against infection of the respiratory tract.

Several investigators compared the immunity induced in chickens by various types of inactivated ND vaccine. Hofstad [30] observed no significant difference between five vaccines containing alumina gel, ethylene glycol, or mineral oil as an adjuvant, with the exception that the percentage of chickens that were resistant in the group vaccinated with one of the alumina gel vaccines was significantly lower than in groups of chickens given the other four vaccines.

Jezierski [32] used equal volumes of macerated chicken embryo tissue and aluminum hydroxide (Waldman) in preparing ND vaccine. He compared the immunizing effect of this vaccine with two similar vaccines containing either potassium alum or sodium borate. Chickens injected with any one of the three vaccines resisted a 10^4 to 10^5 challenge dose of NDV. Cordier *et al.* studied the amount and duration of immunity in chickens injected with the inactivated vaccines adsorbed on a superactive prolana carbon No. 200, aluminum, or vaccine containing virus in a fatty base.[3] Birds vaccinated with one of these vaccines resisted challenge by cohabitation and by intramuscular challenge one, three, and six months after vaccination. Coronel [7] found that alumina cream-adsorbed vaccine was more potent than that containing glycerin. In contrast to these findings of Coronel, Skoda and Zuffa [54] obtained better results with vaccine containing a lipoid adjuvant.

Sullivan *et al.*[47] compared the immunizing potency of the three inactivated ND vaccines prepared with GB-Texas, Roakin-NJ, or Manhattan-Kan strains of NDV containing ethylene glycol as an adjuvant. As reported by these authors, no significant difference could be observed in the various groups of vaccinated chickens, irrespective of the strain of the virus used in the vaccine.

Zuydam [61] listed the advantages and disadvantages of adjuvant-containing vaccines. Fabricant [14] expressed a view suggesting that killed ND vaccines containing various adjuvants had proved themselves capable of producing a significant and practical response by accepted immunological criteria when a proper regimen of double vaccination was carried out. According to the author, vaccination with killed vaccines was the only means for the successful eradication of ND.

MATERIALS AND METHODS

Experimental Birds.—Four groups of 450 birds each were used in this experiment. The majority of chickens were standard-bred New Hampshires.* Small numbers of Single-Comb White Leghorns, Silver Cornish crosses, Leghorn-Red crosses, Black Minoreas, and Australorps were mixed with the New Hampshire chicks when they were placed under the electric brooders at 1 day of age. Although all chicks came from dams vaccinated with a commercially prepared, formalin-inactivated, aluminum hydroxide-adsorbed ND vaccine, significant quantities of serum-neutralizing and hemagglutination-inhibiting (HI) antibodies were not demon-

* Obtained from USDA Animal Husbandry Research Division.

strated when pools of serums from 25 1-day-old chickens were assayed.

Facilities.—Throughout the course of this experiment, the birds were housed in four separate 15- by 25-ft. frame buildings with concrete floors. A litter of wood chips or sawdust was maintained at all times. The experimental vaccines were produced and evaluated in the Newcastle Disease Building, a secure isolation unit designed for work with virus diseases of poultry.

Strains of Newcastle Disease Virus.—The two strains of NDV used were the GB-Texas-1948 strain* and the New Texas 2-156 strain.† In this laboratory, the GB-Texas strain of NDV was passed twice in 9- to 11-day embryonating chicken eggs, harvested, and kept in sealed vials in a CO_2 ice chest. When tested, it conformed to characteristics established for this strain at the Wisconsin Newcastle Disease Virus Repository. When the New Texas 2-156 strain of NDV was received in this laboratory, it represented the second embryo passage of a field strain of NDV isolated from an acute outbreak. It was passed once in 10- to 11-day embryonating chicken eggs and kept sealed in a CO_2 ice chest. When subjected to characterization studies, it showed all the characteristics of a velogenic strain of NDV with a marked enterotropism.

Newcastle Disease Vaccines.—The GB-Texas strain of NDV was used in preparing three types of killed ND vaccine. A 10^9 dilution of seed virus was inoculated into 10- or 11-day embryonating chicken eggs, which were then placed in an egg incubator at 99 F. All eggs were candled 24 hours after inoculation, and those containing dead embryos were discarded. The remaining eggs were candled 40 hours after inoculation. The 10^9 dilution of the GB-Texas strain produced 100 per cent embryo mortality in less than 40 hours. All dead eggs were removed from the incubator, chilled in the refrigerator, harvested, and collected in sterile containers. Albumen was discarded at the time of harvesting. The harvested material was then ground in an Eppenbach mill, set at 76, for three minutes. The resulting tissue suspension was filtered through several layers of sterile gauze, and the filtrate was collected in 500-cc. bottles. Portions of this material were obtained for sterility tests and virus titrations. The material collected was treated with a sufficient amount of a 10 per cent solution of *beta*-propiolactone (BPL)** to yield a final concentration of 0.1 per cent. Inactivation was accomplished at 70 F. in two hours.

After inactivation, the material was mixed with one of each of the three adjuvants so that each bottle contained a different adjuvant substance. The adjuvants and their concentrations were as follows: (1) Crystal violet-ethylene glycol solution (1 Gm. crystal violet dissolved in 400 ml. ethylene glycol) in a final concentration of 20 per cent; (2) Amphojel,‡ aluminum hydroxide gel, in a final concentration of 10 per cent; (3) phosphorylated hesperidan§ in a final concentration of 4 per cent.

The macerated tissue containing phosphorylated hesperidin as an adjuvant was emulsified in a Waring blendor for two minutes. All vaccines were kept under refrigeration.

Safety tests on the finished products included bird and embryo inoculations.

EXPERIMENTAL PROCEDURE

Vaccination Program.—At the age of 1 day, 1,800 chickens were allotted to four main groups and placed in separate houses. When they were 14 days old, all chickens, except those maintained in the control group, were vaccinated with one of the three experimental vaccines. Revaccination of these identical groups was accomplished when the birds were 12 weeks old. In both vaccinations, 0.5 ml. of vaccine was injected into the subcutaneous tissue of the neck.

Serological Tests.—Prevaccination blood samples were taken from chickens when they were 1 and 14 days old. Postvaccination blood samples were taken from 5 chickens, selected at random in each group including the control group, when 4, 6, 8, 10, 12, 14, 16, 20, 24, 28, and 32 weeks old. A total of 250 samples was obtained. The samples were allowed to clot, and the serum was collected, filtered through Boerner centrifugal filters, and stored in the refrigerator until tested. With the exception of the pooled samples obtained when the birds were 1 day old, all samples were collected, stored, and tested separately.

The *alpha* procedure was used to determine the HI titers. The individual values obtained were converted to geometric mean titers. Serum neutralization titers were treated in the same way in the final compilation. The GB-Texas strain of NDV was used in these tests.

Challenge Exposure.—A lot of 20 to 25 birds from each group of chickens which received injections of one of the three types of ND vaccine, as well as a similar lot from the control group, was brought to the Newcastle disease building when the chickens were 4, 6, 8, 10, 12, 14, 16, 20, 24, 28, and 32 weeks old. The immunity of these chickens was challenged with the previously described strains of NDV. When 4 and 6 weeks old, the birds were challenged with the GB-Texas strain only. At the time of the 8-week challenge and later, half of the birds in each lot were exposed to the GB-Texas strain of NDV and the other half to the New Texas 2-156 strain. In all instances, the birds were given 0.1 ml. of a 10^3 dilution of one of the challenge viruses in the muscles of the thigh.

At the same time, the 50 per cent bird lethal

* Obtained from the Newcastle Disease Virus Repository, University of Wisconsin, Madison.

† Obtained from Dr. L. C. Grumbles, head, Department of Veterinary Microbiology, Texas A. and M. College, College Station.

**BPL was supplied by the B. F. Goodrich Chemical Co., a division of B. F. Goodrich Co., Cleveland, Ohio.

‡ Amphojel was supplied by Wyeth Laboratories, Inc., Philadelphia, Pa.

§ Phosphorylated hesperidin was supplied by the National Drug Company, Philadelphia, Pa.

dose (b.l.d.$_{50}$) of each challenge virus was established by injecting unvaccinated control chickens with virus dilutions of 10^6, 10^7, 10^8, and 10^9, using 3 chickens for each dilution. The value was computed according to the method of Reed and Muench. During the course of this study, the GB-Texas and New Texas 2-156 strains of NDV showed only minor variations in b.l.d.$_{50}$, e.l.d.$_{50}$ (50% embryo lethal dose), and hemagglutination (HA) titers.

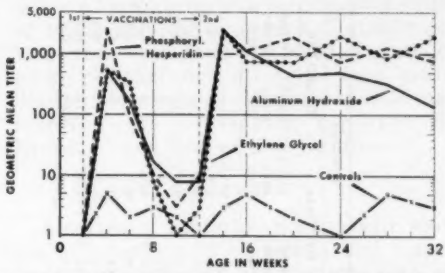
Virus Isolation Attempts.—Attempts were made to recover NDV from the blood of a representative number of birds after challenge. Heparinized blood samples were taken 48 and 72 hours after challenge exposure. Packed erythrocytes were resuspended in broth and used to inoculate 10- to 11-day chicken embryos. Chorioallantoic fluids, obtained from all embryos dying 24 hours or more after injection, were tested for sterility and HA ability. Those showing a positive HA were considered infected with NDV.

RESULTS

The Newcastle disease HI titers obtained in various groups of chickens before vaccination, after the first vaccination, and at later periods are shown (graph 1). Vaccination of 2-week-old chickens resulted in an appreciable rise in HI levels in all groups, as compared with the control group. The anamnestic response obtained in groups of birds subjected to revaccination was similar in all three types of vaccines. The HI titers reached their highest levels when chickens were about 14 weeks old, and showed only slight fluctuation when tested in birds that were 16, 20, 24, 28, and 32 weeks old.

The Newcastle disease SN titers obtained before vaccination, after the first vaccination, and after revaccination are shown (graph 2). Results indicate that the experimental vaccines made with one of three types of adjuvants produced various levels of neutralizing antibodies after the initial injection of vaccine. Significant SN titers were produced in chickens injected with the vaccine containing phosphorylated hesperidin and aluminum hydroxide. Revaccination afforded an appreciable and similar rise in SN titers with all three types of vaccines. These titers persisted at almost the same level until the birds were 32 weeks old, the last age tested. It is evident that the peak of virus-neutralizing ability, like the HI ability, was first reached when the chickens were about 14 weeks old.

Resistance to challenge with a homologous (GB-Texas-1948) and a heterologous (New Texas 2-156) strain of NDV, expressed in percentages of resistance to challenge in

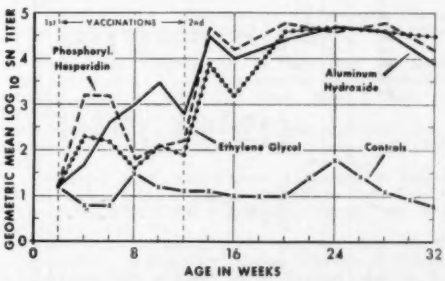


Graph 1—The comparative hemagglutination-inhibition titers of several types of Newcastle disease vaccine (GB Texas strain). First vaccinations were given when chickens were 2 weeks old, and second vaccinations were given when 12 weeks old.

chickens vaccinated and revaccinated with the three experimental vaccines, as well as that of unvaccinated controls, is shown (table 1).

This study indicates that ten weeks after the first vaccination (the last period tested before revaccination), 70 to 90 per cent of birds whose immunity was challenged with a homologous strain of virus resisted challenge. The corresponding values for similar groups of birds whose immunity was challenged with a heterologous strain of NDV ranged from 50 to 90 per cent. Revaccination caused an appreciable increase in the degree of protection, which is evident from the elevated values expressed in percentage resistance. These values ranged from 80 to 100 per cent for those lots of birds whose immunity was challenged with a homologous strain and from 82 to 92 per cent for those whose immunity was challenged with a heterologous strain of NDV when 32 weeks old, the last period tested.

Results of virus isolation attempts after challenge in the three groups of vaccinated



Graph 2—Comparative serum-neutralization titers of chickens given double vaccinations with various types of killed Newcastle disease vaccine (GB-Texas strain) when 2 and 12 weeks old.

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TABLE 1—Results of Challenge Studies and Virus Isolation Attempts Following Challenge with an Homologous or Heterologous Strain of Newcastle Disease Virus

Age of birds (weeks)	Ethylene glycol				Aluminum hydroxide				Phosphorylated hesperidin				Controls			
	GB-Texas		Texas 2-156		GB-Texas		Texas 2-156		GB-Texas		Texas 2-156		GB-Texas		Texas 2-156	
	Resistance (%)	Virus isolation	Resistance (%)	Virus isolation	Resistance (%)	Virus isolation	Resistance (%)	Virus isolation	Resistance (%)	Virus isolation	Resistance (%)	Virus isolation	Resistance (%)	Virus isolation	Resistance (%)	Virus isolation
4	100	1/10*	.....	.....	100	0/10	.....	.....	100	0/10	.....	.....	5	10/10	.....	.....
6	95	1/5	.....	.....	100	0/5	.....	.....	95	0/5	.....	.....	0	5/5	.....	.....
8	100	0/6	80	6/6	100	1/6	80	3/6	90	1/6	60	6/6	0	6/6	10	6/6
10	80	2/6	80	4/6	90	0/6	80	5/6	100	2/6	70	5/6	0	6/6	10	6/6
12	70	2/6	50	6/6	90	0/6	90	3/6	90	3/6	80	6/6	10	6/6	0	6/6
14	100	0/6	100	0/6	100	0/6	100	0/6	100	0/6	100	0/6	0	6/6	0	6/6
16	100	0/6	92	1/6	100	0/6	92	0/6	100	0/6	92	1/6	8	6/6	0	6/6
20	100	0/6	100	0/6	100	0/6	100	0/6	100	1/6	100	1/6	12	6/6	0	6/6
24	100	2/6	92	0/6	100	0/6	92	0/6	100	0/6	100	0/6	0	6/6	13	6/6
28	92	0/6	75	2/6	100	0/6	100	0/6	100	0/6	100	2/6	27	6/6	19	6/6
32	100	0/6	92	0/6	88	1/6	82	0/6	100	1/6	92	2/6	25	4/6	33	6/6

* Numerator = No. of virus recoveries; denominator = total No. of attempts.

and revaccinated chickens and in the unvaccinated control group with a homologous and a heterologous strain of NDV are shown (table 1). In each group of birds whose immunity was challenged with a homologous strain of NDV, 69 virus isolation attempts were made. Of these, eight (11.5%) were found positive in each of the groups of birds vaccinated with the vaccines containing ethylene glycol or phosphorylated hesperidin, and two (2.9%) in the group vaccinated with aluminum hydroxide-adsorbed vaccine were positive. In the control group, 67 (97%) isolations were made. In each group of birds whose immunity was challenged with a heterologous strain of NDV, 54 virus isolation attempts were made. Of those, 17 (31.4%) were found positive in the group of birds vaccinated with a vaccine containing ethylene glycol, 11 (20.3%) in the group injected with aluminum hydroxide-adsorbed vaccine, and 23 (42.5%) in the group vaccinated with a vaccine containing phosphorylated hesperidin. In the control group, 54 (100%) virus recoveries were made.

DISCUSSION

The experiments described in this paper demonstrated the degree and duration of immunity following the use of three types of vaccine prepared with the GB-Texas strain of NDV.

The adjuvant action of phosphorylated hesperidin was clearly demonstrated, since the HI and SN titers afforded by this type of vaccine paralleled the titers of vaccines containing ethylene glycol or aluminum hydroxide. The Newcastle disease HI and SN titers also indicated that none of the groups of birds acquired an absolute immunity that persisted throughout the broiler growing period with a single injection of any one of the three experimental vaccines. Birds given a second injection of vaccine showed a heightened serological response which lasted at least 20 weeks after the second vaccination.

Challenge studies in which a homologous or a heterologous strain of NDV was used revealed higher susceptibility and lower resistance to infection in groups of birds that were exposed to the heterologous strain. This was observed especially in the first phase of the experiment where the groups of birds were singly vaccinated.

Although these results suggest that the heterogenicity of the New Texas 2-156

challenge strain of NDV is totally responsible for the increased susceptibility of vaccinated birds to challenge, it should be pointed out that, in other studies conducted in this laboratory, similar differences in susceptibility were noted in a group of birds injected with a vaccine containing the California 11914 strain of NDV and challenged with either the GB-Texas or the New Texas 2-156 strain of NDV. Additional data on the characterization of the New Texas 2-156 strain of NDV were furnished by necropsy of challenged birds. Tissues obtained from the abdominal tracts of diseased chickens revealed predominantly hemorrhagic lesions, with severe and extensive involvement of the proventricular mucosa characterized by pinpoint hemorrhages, ulcerative foci, and necrosis. Upon histopathological examination, the lesions were found to be confined to the proventricular mucosa and showed congestion, hemorrhage, and large necrotic foci. There also were hyaline changes in many of the arterioles and smaller arteries. Significant changes were not seen in the glandular or muscle layers. All necrotic lesions were of viral origin, but the circumstantial evidence would indicate that the large necrotic foci were the result of secondary bacterial invaders, which were found in large masses in these areas.

Additional data on the immune status of chickens vaccinated with the three types of ND vaccine were sought by the postchallenge virus recovery procedures. Since such techniques were subject to variation resulting from the availability of time and materials, the results obtained must be considered to be of a supportive rather than definitive value. Under the conditions used in this experiment, immunity based on postchallenge virus recovery was substantially lower than immunity indicated by postchallenge mortality. Results obtained in groups of birds after the initial injection of vaccine emphasized the incomplete immune status elicited by a single vaccination. When a booster injection was given, the discrepancy noted between results obtained with the two challenge criteria was found to be modified.

An increased progressive resistance to infection with NDV was demonstrated during development to maturity in the control group. This increasing refractoriness to ND with maturation has been manifested by an increased resistance to challenge with either a homologous or a heterologous strain of virus and by a decreased number of virus recoveries from the blood of chickens when postchallenge attempts were made. Similar observations referring to challenge results were previously reported by Brandly [5] and Waller and Gardiner.[59]

SUMMARY

A review of literature dealing with various adjuvants used in human and veterinary medicine and their mechanism of action was presented.

The degree of immunity induced was compared in groups of chickens vaccinated with three types of inactivated Newcastle disease vaccines prepared with the GB-Texas-1948 strain of Newcastle disease virus. The adjuvants studied included ethylene glycol, aluminum hydroxide, and phosphorylated hesperidin. The immune status of the birds was determined after primary vaccination when the chickens were 2 weeks old and after revaccination when 12 weeks old. Criteria used to evaluate the immunity of chickens at various ages, 4 to 32 weeks old, included the hemagglutination-inhibition and serum-neutralization tests, challenge studies, and postchallenge virus isolation attempts.

The results of these investigations showed that the immunogenicity of vaccines made with phosphorylated hesperidin, ethylene glycol, or aluminum hydroxide was similar.

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The Pathology of Canine Dirofilariasis

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THE PATHOGENESIS of canine dirofilariasis, or heartworm disease of dogs, is not well understood. In this it resembles other filarial infections in man and other animals.

The demonstration of the life cycle of *Dirofilaria immitis* is generally attributed to Fülleborn,[11] although its earliest elucidation was made by Bancroft [4, 5] in Australia. Since then, the parasite and its habits have been studied extensively and often compared with *Wuchereria bancrofti*, a related filaria found in the lymph vessels of man. Various species of mosquitoes are suitable intermediate hosts,[15, 29, 30] but there is a conflict of opinion as to whether other blood-sucking anthropods, like fleas and ticks, are suitable for the development of infective larvae.[7, 8, 16, 23, 26, 27, 28] The development of the parasite within the host from the time of the infection until the occurrence of adults has only recently been studied successfully by Kume and Itagaki,[17] who showed that, after infection, the larvae spend part of their developmental stages in the subcutaneous, adipose, and muscle tissue and in the submuscular membrane and subserosa.

The clinical signs of canine dirofilariasis are subject to considerable variation. [2, 9, 14, 24] There is a tendency to tire easily, listlessness, loss of weight, unthrifty coat, marked coughing after exercise, sometimes dermatitis, anemia, dejected appearance and, occasionally, emaciation. Dogs with heavy infections of long standing may show ascites, as well as edematous swelling of the ventral thoracic wall and extremities. Demonstration of microfilariae in the blood is not always successful. However, microfilariae may be present in the blood when no adult worms are found in the heart.[10] Armistead [3] attributes this to the presence of adults in other parts of the body. *Dirofilaria immitis* has, for instance, been removed from the eye, an interdigital cyst,[25] and the abdominal cavity.[9]

Recently, in the United States, Newton and Wright [19, 20] discovered a wide distribution of a species of Dipetalonema, believed to be *Dipetalonema recondita*, a filaria which parasitizes the subcutaneous tissue of the dog. The microfilariae show morphological differences from those of *D. immitis*, but these may easily be overlooked by an examiner not aware of the presence of two different species. These new findings should stimulate renewed and more successful studies of various aspects of the disease, such as survival of larvae in the blood and the intermediate hosts, as well as the relationship between numbers of microfilariae and adults. *Dipetalonema recondita* has been described by Grassi and Calandruccio,[12] but since then has been more or less forgotten, probably because the adults, being only one tenth the size of *D. immitis*, are difficult to detect in routine necropsies. Intermediate hosts appear to be cat and dog fleas (*Ctenocephalides canis* and *Ctenocephalides felis*).

The study of the pathological aspects of the disease to complement and interpret clinical observations has been neglected. Reports of necropsy findings describing anything more than the presence of parasites in various locations are rare.[1, 21] Living worms are found in the right side of the heart and dead worms in the pulmonary artery. When a large number of worms is present, they may also be seen in the hepatic vein. In chronic cases, cardiac dilatations, pneumonia, hepatic congestion, ascites, and subcutaneous edema have been mentioned. Pollock [22] gave a more detailed report of a 5-year-old dog which, on macroscopic examination, showed numerous heartworms and passive venous congestion of the liver. Histological sections revealed numerous macrophages in the lungs containing either lipoid or blood pigment and cross sections of adult parasites. The spleen contained many siderocytes and plasma cells. The kidneys showed marked albuminosis. The liver was severely congested and there was bile retention.

The damaging effects of *D. immitis* are generally attributed to the mechanical interference of the adult parasites with the host's circulation. Toxic effects by adults or larvae are considered unlikely,[21] although Bozicevich and Hunter [6] found some indication of toxicity of this parasite when 7 of 12 rabbits died after having been injected with the residue obtained from antigen preparation. Adams [1] found various inflammatory reactions in the lungs of an animal which he attributed to the "numerous microfilariae diffusely distributed in the parenchyma."

In general, it can be said that our knowledge of the parasite's zoological aspects, morphology, and life cycle is reasonably good. In contrast, the disease produced by the parasite is not well understood. Clinical signs are known, but to attribute them to the mechanical interference of the parasite with the circulation does not satisfactorily explain many of them, especially those in chronic cases and particularly their unsuccessful treatment.

Heartworms are found in about 10 per cent of the dogs necropsied at the Univer-

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sity of Queensland Veterinary School. In the course of these examinations, 12 dogs were fully studied histologically.

The present study is concerned with undescribed lesions that have been found and their significance and relationship to clinical manifestations.

Observations

In addition to the presence of adult parasites in the heart, characteristically prominent lesions have been found in lungs, lymph nodes, spleen, and liver.

Macroscopic Examination

Heart.—The parasites were found in varying numbers in the right side of the heart, often extending into the pulmonary artery. Slight chronic fibrotic thickening of the atrioventricular valves was frequently noticed. As this occurred in the left and right ventricles of middle-aged and old dogs, and similar lesions are frequently found in uninfected dogs of this age, it is doubtful if they can be attributed to the heartworms.

Lungs.—The lungs were generally of normal or slightly firm consistency and were pale with an unusual rust-brown discoloration. There were also circumscribed, consolidated, irregular, greyish nodules up to a few centimeters in diameter located mainly in the dorsal parts of the lungs. These nodules were associated with parasites and thrombi in the branches of the pulmonary artery. The bronchi contained a slightly increased amount of cloudy mucus; there was no significant edematous change. The described appearance of the lungs and other organs was sometimes camouflaged, particularly by hyperemia or pneumonia, if the dog was affected with a condition causing such changes in addition to dirofilariasis.

Lymph Nodes.—When no other changes but those of dirofilariasis were found in the tributary areas, the lymph nodes in the thoracic cavity constantly showed a brown discoloration of the medulla and a slightly moist cut surface. Other regularly examined lymph nodes which showed the brown color in decreasing intensity were the superficial cervical, axillary, mandibular, and iliac nodes.

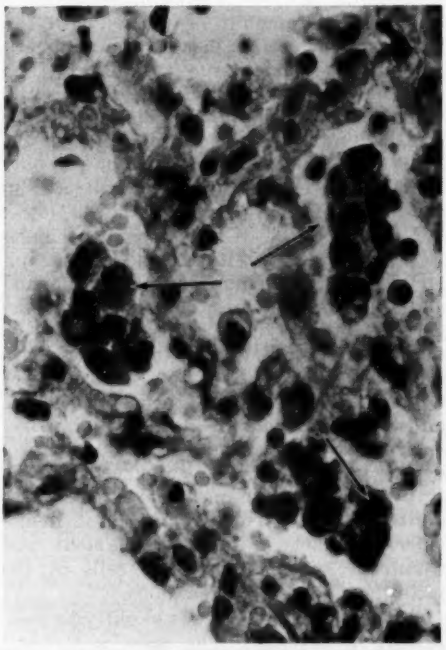

Fig. 1—Siderocytes (arrows) in the lungs of a dog. H & E stain; x 600.

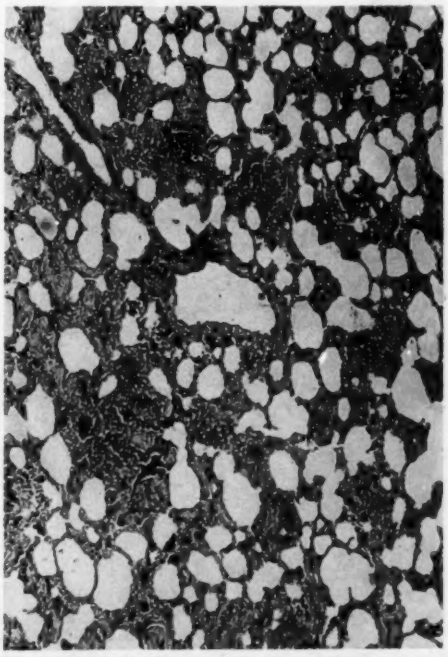

Fig. 2—Pulmonary fibrosis in a dog. H & E stain; x 40.

When the parasites in the heart were numerous, the changes in the lungs and lymph nodes were invariably well developed. In some dogs with only a few heart worms, the changes were pronounced. In others they were of lighter intensity, with few or no thromboses and pulmonary nodules.

Liver.—When the disease had progressed so far that the clinical signs indicated circulatory disturbances, the liver was congested. This congestion resulted in a variation from a moderate enlargement with slightly granulated surface, dark bluish red discoloration, and an increased amount of blood oozing from the cut surface to a greatly enlarged, irregularly shaped liver showing distended lymph vessels, especially near the dorsal parts, and an excessive amount of bleeding from the cut surface. Such an extensively changed liver was always accompanied by ascites, as well as anasarca of the ventral part of the body.

Spleen.—In advanced cases with ascites and anasarca, the spleen was slightly enlarged and of firm consistency.

Kidneys.—In advanced cases, the kidneys showed a brown discoloration, mainly of the cortex.

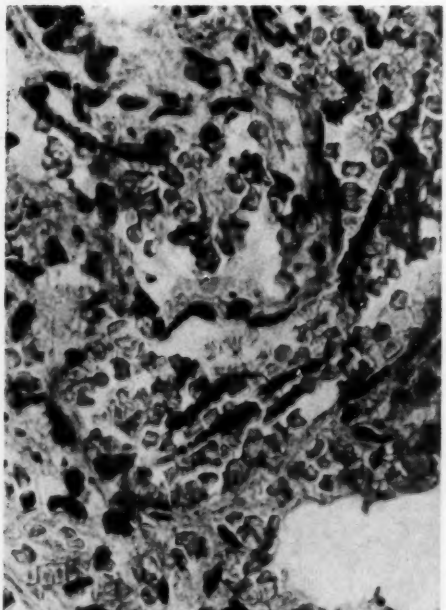

Fig. 3—Microfilariae in blood vessels of lungs of a dog. H & E stain; x 500.

MICROSCOPIC EXAMINATION

Lungs.—The histological sections of the lungs revealed three prominent features, *i.e.*, inflammatory infiltration of various degrees, hemosiderosis, and fibrosis. The inflammation ranged from a slight cellular infiltration to a more severe, predominantly productive reaction.

The outstanding inflammatory cells were macrophages, a large number of which contained brown granulated pigment. Since this pigment gave a positive Prussian blue reaction, it was hemosiderin (fig. 1). These siderocytes were found in the alveoli, as well as in the interstitial tissue, and particularly around larger blood vessels. Numerous macrophages were without pigment or contained a few to numerous fat vacuoles. Numerous macrophages without pigment, as well as smaller numbers of neutrophils, plasma cells, and a few eosinophils, formed the nodules around thrombosed arteries. Here the walls of the blood vessels showed erosion and inflammatory infiltration, and the lumen contained thrombi and sections of parasites. The bronchi contained an increased amount of mucus with few inflammatory cells, mostly macrophages. The interalveolar tissue was fibrotic and thickened in chronic cases and especially when ascites and anasarca had developed (fig. 2). Microfilariae were found in the blood vessels of all sectioned tissues in only 1 dog and were markedly concentrated in the lungs (fig. 3).

Lymph Nodes.—Characteristic changes were found in the sinuses and the medullary cords, particularly in the bronchial and mediastinal nodes, and to a lesser degree in other lymph nodes. There were numerous erythrocytes in the sinuses, both free and phagocytized by macrophages. Other macrophages contained hemosiderin, and a few harbored granules of hemosiderin, as well as one or several erythrocytes (fig. 4). The reticular cells in the sinuses and in the medullary cords also contained numerous granules of hemosiderin. Black anthracotic pigment was found in enlarged reticular cells of the medullary cords of a few dogs.

Spleen.—The spleen was slightly to moderately hyperemic. The most significant change was the presence of hemosiderin,

ranging from slightly increased to extensive pigmentation. The level of hemosiderosis in the spleen corresponded to that of the lungs, although it never reached the same intensity.

Liver.—In chronic cases with clinically detectable circulatory disturbances, the liver showed passive venous congestion. Congestion varied from a moderate dilatation of the central veins and surrounding sinusoids and occurrence of fat vacuoles in centrolobular liver cells to an extensive dilatation of the blood vessels causing pressure atrophy of the parenchyma, so that islands of liver cells remained mainly around the portal triads. Numerous dilated lymph vessels were then seen under the liver capsule. The Kupffer cells contained hemosiderin in the nonhyperemic areas, but hardly more than expected in a normal liver. It seemed that the more the liver was congested, the less hemosiderin it contained.

Kidneys.—In 1 of 12 dogs examined histologically, the kidneys showed a significant infiltration of the epithelial cells of the convoluted tubules with granules of hemosiderin. Of all the dogs examined, this animal showed the highest degree of pigmentation.

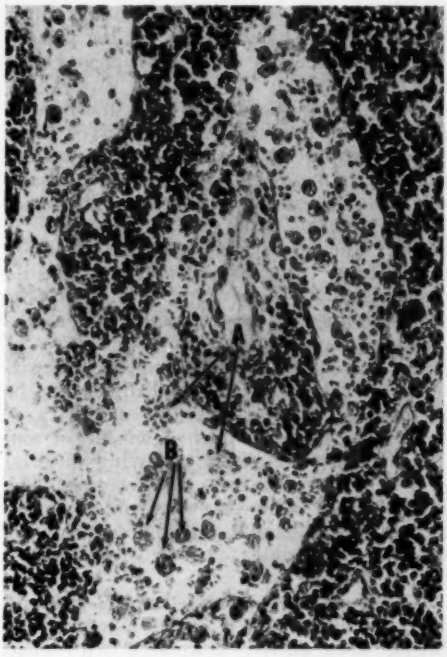

Fig. 4—Medullary sinus of bronchial lymph node of a dog, showing erythrocytes (A) and macrophages (B). H & E stain; x 133.

Discussion

The outstanding lesions which apparently were related to clinical signs were: in the lungs, hemosiderosis, chronic inflammation, thrombosis, fibrosis, and moderate emphysema; in the lymph nodes, erythrophagia and hemosiderosis; in the spleen, hemosiderosis; and in the liver, passive venous and, in advanced cases, lymphatic congestion with relatively little hemosiderosis as compared to other organs. Further lesions were ascites and anasarca.

Increased accumulation of hemosiderin indicated an increased destruction of erythrocytes. Most of the hemosiderin was found in the lungs and their regional lymph nodes, less in other lymph nodes and spleen, and little in the liver. This distribution indicated that the site of erythrocyte damage was somewhere in the right side of the heart, the pulmonary artery, or the lungs. It is known that pulmonary hemosiderosis may be caused by chronic venous congestion in the course of heart failure. In typical cases of dirofilariasis, however, the lungs are anemic, rather than hyperemic.

So far, there is no indication of toxicity of the parasite which could be responsible for the destruction of blood. No morphologically changed erythrocytes were seen in the tissues and sinuses of the lymph nodes. If a toxin caused hemolysis, a relatively higher degree of hemosiderosis could be expected in the spleen. It is also unlikely that dead, disintegrating adults are responsible for hemosiderosis, as the nodules caused by them contained almost no pigment. These observations and the concentration of hemosiderin mainly around larger blood vessels indicated that iron-containing waste products of the adult's digestion are mainly responsible for the pulmonary hemosiderosis. This is supported by observations of blood in the intestines of *D. immitis* made by Mackerras.[18]

Hemosiderosis of the lungs must also be partially responsible for hemosiderosis of the regional lymph nodes. However, part of the pigment in the lymph nodes is undoubtedly derived from the erythrocytes which are found in the sinuses, both free and in phagocytes. Lymph nodes, other than those regional to the thoracic organs,

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as well as the spleen and kidneys must have received hemosiderin or its precursor from material which had passed into the blood stream.

Considering the unlikeliness of toxic effects of the parasites, blood derived from the intestinal tract of the parasites also appears to be responsible for the inflammation of the lungs. This is further supported by the type of inflammatory reaction. The agent causing the inflammation is not toxic enough to stimulate a significant vascular reaction and exudation. The predominance of macrophages indicates a chronic process, calling for phagocytosis of irritating material of no significant toxicity, probably too large and difficult to be handled by neutrophils. The majority of the macrophages contain hemosiderin and are therefore engaged in removing material originally derived from erythrocytes. The inflammatory cells removed from the bronchi with an increased amount of mucus are mainly responsible for the cough noticed in the disease.

The greyish, consolidated nodules, found predominantly in the dorsal parts of the lungs, were always associated with dead adults embedded in thrombi in the pulmonary artery. Here, also, the inflammatory reaction gave the impression that the organism was chiefly concerned with the removal of a mechanically irritating foreign body. The occasional eosinophils indicate that there is little, if any, of the allergic reaction often associated with parasites. Lesions of atherosclerosis, comparable to those frequently encountered in man and apparently observed by Hermigar and Ferguson,[13] could not be found.

In the further course of the disease, the inflammation, like most chronic inflammations, resulted in fibrosis and was accompanied by moderate emphysema. This was due to the degeneration of elastic fibers in the course of the inflammation. Pneumatic pressure was responsible for the mechanical factor, a principle known in other lesions such as aneurysm and bronchiectasis.

So far, mechanical interference of the adult parasites with the action of the heart and the lumen of the pulmonary artery has been blamed for the circulatory disturbances. This, however, does not explain the poor clinical response to therapy in chronic cases, even when all adult parasites have been destroyed. It appears that in long-standing infections, especially in those leading to ascites and anasarca, pulmonary fibrosis and moderate emphysema are the main and permanent obstacles for the circulation. Mechanical interference of the parasites constitutes a temporary and removable obstruction. This must be the reason why, in a dog which has already developed ascites and anasarca, the prognosis for recovery is poor.

The relatively small amount of hemosiderin in the liver of the dogs studied is probably due to its reduced efficiency and overburdening resulting from venous congestion.

SUMMARY AND CONCLUSIONS

The disease caused by *Dirofilaria immitis*, the heartworm of dogs, is not well understood because of the lack of studies of pathological aspects to complement and interpret the clinical observations.

These parasites were found in about 10 per cent of the dogs necropsied at the Veterinary School, University of Queensland. The prominent lesions observed were: hemosiderosis, chronic inflammation, thrombosis, fibrosis, and moderate emphysema in the lungs; erythrophagia and hemosiderosis in the lymph nodes; hemosiderosis in the spleen; passive venous and, in advanced cases, lymphatic congestion in the liver; and ascites and anasarca.

The lesions and their relationship to clinical manifestations indicated that no toxin was produced by the parasites. The pulmonary hemosiderosis and inflammation probably were caused by the waste products of the adults' digestion.

In chronic cases, the inflammation resulted in pulmonary fibrosis with moderate emphysema; this was mainly responsible for the irreversible circulatory disturbances, the development of ascites and anasarca, and the poor prognosis in advanced cases.

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A Comparison of Uric Acid Determinations and Sulfobromophthalein Sodium Retention Tests as an Index to Canine Liver Dysfunction

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IN RECENT YEARS much has been written about the liver and liver function tests. Misconceptions have resulted from some of these reports, particularly with regard to the application of certain tests in veterinary medicine. The purpose of this study was to investigate some of the controversial tests, evaluating their results when used on the dog.

Since blood and urine uric acid levels have been mentioned many times as an index of hepatic function on the dog, and since the sulfobromophthalein sodium (Bromsulphalein,* BSP) retention test has had widespread use, it was decided that a comparison would be made of these three tests on dogs having known liver dysfunction.

REVIEW OF LITERATURE

Uric acid was discovered in 1776.[24] Further information of the phenomena which serves as a basis for liver testing in animals was not discovered for about 100 years. The basic principle is that most mammals, except man and some monkeys, metabolize purines to uric acid and finally to allantoin. An enzyme from the liver, uricase, is necessary for conversion of uric acid to allantoin. According to Folin *et al.*,[13] uric acid metabolism in the dog differs so much from the process in man that it seemed scarcely worthwhile to study it in the dog. However, research was stimulated by the discovery, by Benedict,[2] that the Dalmatian dog excretes uric acid in a manner similar to man. Considerable research was done to solve the problem of why one breed of dogs metabolized purines differently from others.[25, 26, 43, 44] Klemperer *et al.*[25] clouded the issue by demonstrating that the liver of the Dalmatian dog is rich in uricase, and that no correlation could be found between the amount of uricase present in the liver and the destruction of uric acid *in vivo*.

Friedman and Byers[14] investigated the problem of increased uric acid excretion in the Dalmatian dog and found that it converted blood uric acid into allantoin less readily and completely than did the non-Dalmatian. They considered this only a minor effect when compared with the over-all picture, but it does explain the higher blood plasma levels of uric acid in Dalmatians. They found that the principal cause of greater amounts of uric acid in Dalmatian urine was that uric acid is filtered by the glomerulus and is not reabsorbed by the tubules. They substantiated their work by showing that both Dalmatian and non-Dalmatian dogs produce and excrete the same total amounts of purine end products (uric acid and allantoin).

The use of uric acid levels as an index of liver activity has been suggested but not substantiated. Bloom[3] considers the blood uric acid level of dogs to be between 0.0 and 1.0 mg. per 100 ml. He stated that: "The determination of the blood uric acid is a sensitive and accurate indicator of liver disease, since even relatively mild liver damage is accompanied by increased blood levels."

In another writing, Bloom[22] stated: "While there are many tests for hepatic function, the most accurate is estimation of blood uric acid in the dog. This substance is invariably elevated in liver hypofunction of clinical degree." Morris[35, 36] considers estimation of urine uric acid to be quite accurate as an indicator of liver disease. He believes that an increase of uric acid excretion over 5 mg. per kilogram of body weight per day to be indicative of liver dysfunction.

Sulfobromophthalein Sodium Retention Test.—In 1925, Rosenthal and White[41] first reported the use of BSP as a test of liver function. Their decision to use this dye resulted from several years' experimentation with other phthalein dyes. The technique devised by Rosenthal and White required that the dye be given intravenously to a patient at the rate of 2 mg. per kilogram of body weight. Thirty minutes after injection, a sample of blood was obtained from the opposite arm. Serum was obtained from the blood, and the amount of dye remaining in the blood stream was estimated by comparing the serum with previously prepared standards. After 30 minutes, the blood stream was expected to be entirely free from dye.

Following its introduction, BSP gained in popularity as a liver function test. O'Leary *et al.*[39] made the first significant modification in 1929 when they proposed a dose of 5 mg./kg. of body weight

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* Bromsulphalein is the trade name of sulfobromophthalein sodium, U.S.P. Hynson, Westcott and Dunning, Inc., Baltimore, Md. Material for this study made available through the courtesy of H. B. Dunning.

instead of the 2 mg./kg. of body weight as originally used by Rosenthal and White.[41] Israel and Reinhold[24] used the same dose, and analysis was still made of a blood collection drawn 30 minutes after injection. Helm and Machella[19] compared various methods and concluded that Israel and Reinhold's was the most satisfactory. Mateer *et al.*,[21] in 1943, suggested the use of a 5 mg./kg. dose with no retention after 45 minutes in the absence of liver damage. This is the dose and the time interval most used in human medicine today.

There was limited use of BSP in veterinary medicine for many years; however, considerable testing was done on animals by physicians for application to human medicine. McKibbin *et al.*,[20] in 1943, suggested a modification for use on dogs which required a dose of 7 mg./kg. of body weight and a sample being obtained after eight minutes. Percentage standards were not used, but it was assumed that significant color after eight minutes indicated liver damage. The method apparently was not accepted, for it was not mentioned in other literature. Drill and Ivey[11] used a dosage of 5 mg./kg. of body weight with a 30-minute withdrawal time in a comparison of BSP with other tests on dogs whose livers had been damaged with carbon tetrachloride. They considered retention in excess of 15 per cent indicative of liver damage. A comparator block set of standards was used in evaluation, and accurate determinations of trace amounts was difficult, since the blocks used were designed for a dose of 2 mg./kg. of body weight. When higher dosages were used, much higher readings were obtained.

Using essentially the same method suggested by Drill and Ivey, Hoerlein and Greene[23] made serial withdrawals from 10 normal dogs in an effort to establish the best withdrawal time. They concluded that anything greater than a trace at 30 minutes indicated liver damage.

Hemolysis is a constant problem in collecting canine blood and will interfere greatly with BSP methods. In an effort to overcome this problem, considerable work has been directed toward compensation for hemolysis in plasma or serum. Gaebler[15] used spectrophotometric methods to compensate for turbidity, hemolysis, and icterus. Cantarow and Wirts[7] had previously used photometric methods for determination of BSP in bile. The method used by Gaebler[15] was similar in many respects to a method described by Hawk *et al.*,[18] which, while apparently quite accurate, required considerable time and effort in calculation. A simpler method, and one that is practically identical with the original work of Rosenthal and White, was suggested by Popper and Schaffner.[40] It requires that 2 ml. of serum be diluted to 10 ml. with 0.85 per cent saline solution. The mixture is equally divided, and sodium hydroxide is placed in one portion, while hydrochloric acid is placed in the other. The alkaline solution, which shows the BSP, is compared to the acid solution at a wave length of 580 mμ. This method seems to be most suited in canine testing because of hemolysis which frequently occurs in canine blood samples.

Little work was done to determine why BSP is effective as a liver function test until after 1945, when considerable attention was given to factors which alter its disappearance from the blood stream,[5, 25, 33] the rate of removal of BSP by extrahepatic tissues,[9] the effect of enterohepatic circulation on the test,[30, 39] and clearance methods.[16, 27-29, 37] While research continued, agreement was still lacking on the exact route BSP travels before being eliminated in the bile. A preparation of S^{35}-labeled BSP was used by Brauer *et al.*[6] in an attempt to trace its pathway. They concluded that definite changes occurred in the BSP molecule and that some were lost by destruction. They also demonstrated that the muscles of the body acted as the chief site of extrahepatic dye retention but concluded that errors due to extrahepatic activity were small. Brauer *et al.* stated that: "Almost 80 per cent of the total dye removed from the circulation in normal dogs can now be accounted for in terms of hepatic extraction." Bradley and his associates[4] found that most BSP injected combined with the albumin fraction of serum proteins. This was in agreement with the findings of Cohen *et al.*,[9] who further demonstrated that some BSP combines with with serum globulin.

When BSP is attached to serum proteins, it circulates until it is released by the liver for excretion in bile. Interference with that release, resulting in continued circulation, is the basis for diagnosis of liver dysfunction. This test is recognized by many workers as the most sensitive test of liver function.[10, 17, 31, 42]

MATERIALS AND METHODS

Uric Acid Determination.—Three different methods were used for blood uric acid determinations; however, the method of Kern and Stansky, as modified by Archibald,[1] proved to be most satisfactory. The method required that 2.0 ml. of plasma be added to 16.0 ml. of distilled water. This was mixed well, and 0.8 ml. of 0.5N sodium hydroxide solution was added to it. The blood proteins were precipitated with 1.2 ml. of phosphotungstic acid, and after five minutes a supernatant fluid was obtained by centrifugation. Five milliliters of the supernatant fluid was placed in an 18- by 150-mm. round cuvette. At the same time, a 5.0-ml. quantity of distilled water and a 5.0-ml. quantity of dilute uric acid standard were placed in similar cuvettes. The three cuvettes containing water, standard, and unknown, respectively, were then treated in like manner for the remainder of the test. To each was added successively a 2.5-ml. quantity of silicate-glycerol reagent and a 2.0-ml. quantity of mixed uric acid reagent. The solutions were well mixed, and after 15 minutes (to allow for maximum color change) the standard and unknown solutions were compared to the blank solution at a wave length of 700 mμ in a Coleman Universal Spectrophotometer.* Each 5.0 ml. of the

* Coleman Universal Spectrophotometer is manufactured by Coleman Instruments, Inc., Maywood, Ill.

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standard solution used contained 0.025 mg. of uric acid.

In calculation of results, the following formula was used:

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times 5 = \text{mg. uric acid/100 ml. of plasma}$$

The normal range for the dog was considered to be 0.0 to 1.0 mg./100 ml. of plasma.

The urine uric acid determination was by a similar method as outlined by Hiller.[20] It varied only slightly and could be run at the same time as the blood method.

A 1.0-ml. quantity of urine was diluted to 100 ml. with distilled water. After thorough mixing, 5.0 ml. was placed in an 18- by 150-mm. round cuvette and treated along with, and exactly the same as, the supernatant fluid from the plasma samples. Calculation varied in that the following formula was used:

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times 2.5 \times \frac{\text{ml. of total urine volume for period of collection}}{\text{ml. of diluted urine used}} = \text{ml. uric acid for period of collection}$$

Calculations were then made on the basis of the quantity of uric acid eliminated per kilogram of body weight. By this method, the upper limit of normal in the dog (regular diet) was considered 10 mg./kg. of body weight per day, but may vary greatly.

Dogs were maintained in metabolism cages and urine was collected and measured daily. Ten milliliters of 5 per cent sodium hydroxide, as recommended by Dubbs *et al.*,[12] was added to the urine collection pan after each emptying to prevent precipitation of urates and growth of bacteria.

Sulfobromophthalein Sodium Method.—Four variations of the BSP test were attempted before it was decided that the following one, most of which was recommended by Popper and Schaffner,[40] was best suited for canine testing.

The amount of dye to be injected was calculated on the basis of 5.0 mg./kg. of body weight. Approximately one minute was allowed for injection, and the mean injection time was considered zero time (midpoint of total injection time was considered mean injection time). After exactly 30 minutes, approximately 6.0 ml. of blood was withdrawn from the opposite side of the body and carefully placed in a clean, dry test tube. Syringes used for collection of blood were lined with heparin (1,000 U.S.P. units per milliliter), since this was found to be the best prevention against excessive hemolysis. After centrifugation, 2.0 ml. of plasma was diluted with 8.0 ml. of 0.85 per cent saline solution. This was well mixed and then equally divided. To 5.0 ml. of the mixture was added a 0.2 ml. sample of 10 per cent sodium hydroxide solution to bring out the alkaline BSP color, and to the other 5.0 ml. was added a 0.2 ml. sample of 10 per cent hydrochloric acid to compensate for any hemolysis present. Both samples were placed in individual cuvettes and read in a Coleman Universal Spectrophotometer at a wave length of 580 mμ using the acid-containing tube as the blank. The readings were made in terms of optical density and compared to a previously prepared standard curve. Results from the standard curve were read as milligrams of dye per 100 ml. of plasma and, when multiplied by 10, gave the percentage retention of the total amount that had been injected. The method used to establish the standard curve was as recommended by Hawk *et al.*[18] A retention greater than 5 per cent after 30 minutes was considered abnormal in the dog.

Method of Producing Liver Damage.—Four routes of hepatotoxin administration (intravenous, subcutaneous, intramuscular, and oral) were used in attempts to produce a chronic liver disorder. The oral route was selected as the best, and the method used was as recommended by Drill and Ivey.[11] A dose of 0.5 ml. of carbon tetrachloride per kilogram of body weight was mixed with an equal volume of corn oil and administered by stomach tube. The dose was given twice each week. A ¼-inch polyethylene tube was used as a stomach tube and a syringe was used to administer the mixture.

EXPERIMENTAL PROCEDURE AND RESULTS

Dogs 1 and 2 were used in development of the BSP test and carbon tetrachloride poisoning methods. Thirty clinic dogs were used in development of blood and urine uric acid test methods. Dogs 3 through 8 were experimentally subjected to all of the tests being studied.

Adult mongrel dogs of 10 to 15 kg. body weight were chosen for experimentation. They were observed for at least one week, and tests were run before any attempt to damage the livers was made. If, after one week, they seemed healthy and responded normally to liver tests, carbon tetrachloride poisoning was initiated. Dogs 3 through 8 were treated in the same manner, and the findings were essentially the same on all of the dogs. To avoid unnecessary repetition, the results obtained from dogs 7 and 8 are reported for comparative purposes and are considered typical of results obtained from the other dogs.

Dogs 7 and 8 were observed and tested for ten days before carbon tetrachloride was administered. Dog 7 had an average blood uric acid level of 0.44 mg./100 ml. of plasma, an average urine uric acid excretion of 6.3 mg./kg. of body weight per day, and an average BSP retention of four tests of 3.7 per cent after 30 minutes. Dog 8 had an average blood uric acid level of 0.39 mg./100 ml. of plasma, an average urine uric acid excretion of 7.2 mg./kg. of body weight per day, and an average BSP retention of 3.4 per cent after 30 minutes.

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Carbon tetrachloride was given on Tuesday mornings and Friday afternoons, which spaced all doses at or near 84-hour intervals. Diagnostic tests were made on Monday, Wednesday, and Friday. Urine samples were collected daily between 9 and 10 a.m. Total daily urine samples were tested for uric acid content. Urine collected on nontest days was run the next day of test (Monday, Wednesday, or Friday). No variation was noticed on alkalinized urine samples as a result of having been held for any period of time.

The normal pattern was followed on both dogs (graphs 1, 2) for all tests during the first four days following initial administration of hepatotoxin, except for a 6 per cent BSP retention on dog 7. On the fifth day, both dogs showed some BSP retention and, during the remaining time the dogs were on experiment, the BSP was not within normal range again.

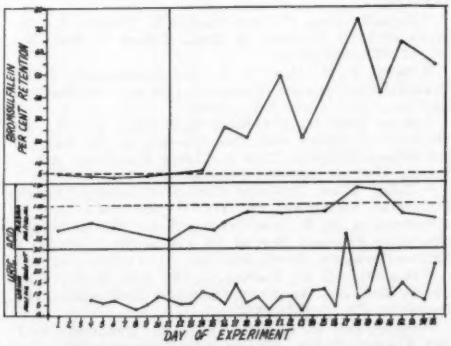
The blood uric acid values began a slow climb, but only in dog 7 did they exceed the normal range. Three tests were above normal, the highest being 1.40 mg./100 ml. of plasma. The first time the normal was exceeded was on the twelfth posthepatotoxin day. In addition, at the time of the highest blood uric acid level, the BSP retention was 74.5 per cent.

Urine uric acid excretion could never be considered excessive, except during the high blood uric acid phase on dog 7 mentioned above. Values were unpredictable, however, and sometimes varied as much as 20 to 30 mg. on successive days.

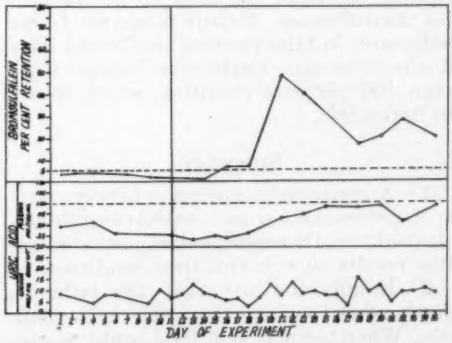
DISCUSSION

A research project concerned with an organ as complex as the liver can never be considered complete. It was originally planned as a comparison of an accepted reliable test with a much-talked of, but relatively nonevaluated, test (uric acid). Before any comparative testing could be made, it was necessary that each test concerned be properly evaluated to ascertain that the best possible results were being derived. In conjunction with this, it was necessary to develop a slow chronic, controllable form of liver disorder so that standard evaluation could be made.

It is believed that the final methods used are the best at the present time for animal testing and also that the comparative results obtained are worthwhile. Although both blood uric acid and urine uric acid show slight increase with progression of disease (graphs 1, 2), the results are seldom more than a high normal. Also, while abnormality of uric acid levels are being approached, the BSP retention is extremely high. Of the 6 dogs reported, only dog 7 exceeded 1.0 mg./100 ml. on blood uric acid at any time, and this occurred nine days after BSP indicated damage. Urine uric acid on these dogs never showed significant rise with the exception of three days on dog 7. But, on the day before and after each of those three peaks, the uric acid excretion



Graph 1—Comparison of daily tests of dog 7. Broken lines indicate normal test figures of sulfobromophthalein sodium and blood uric acid. Normals for urine uric acid on purine-containing diet are not available. Solid vertical line on the eleventh day of the experiment indicates initial carbon tetrachloride damage.



Graph 2—Comparison of daily tests on dog 8. Broken lines indicate normal test figures of sulfobromophthalein sodium and blood uric acid. Normals for urine uric acid on purine-containing diet are not available. Solid vertical line on the eleventh day of the experiment indicates initial carbon tetrachloride damage.

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was well within normal limits. Thus, it would seem that urine uric acid values could not be relied upon due to this rise and fall. There is a possibility, however, that catheterization at closely timed 24-hour intervals would give a smoother curve, but the result would also mean a lowering of the peak values. The wide variations obtained in urine uric acid values could well be due to differences in the amount of exogenic purines consumed as food or in the excretion schedule of the animal. This might also have been controlled by placing the animals on a purine-free diet, but another purpose of the experiment was to see if uric acid methods are practical when animals on many different diets are tested.

If it had been found that uric acid determination was a great diagnostic value, it would still be of little value to the veterinary practitioner. The cost of reagents, the complexity of reagent mixing, the short shelf life of some reagents, and the amount of time consumed on the test would make it of academic value only.

Most of the errors with BSP testing can be attributed to excess hemolysis in the test sample. However, most of the hemolysis can be eliminated by careful technique and by using heparin in the syringes for withdrawals.

Other errors in BSP testing have resulted from misinterpretation of standard blocks. Most comparator block standards were designed for a dosage of 2 mg./kg. of body weight and, when a 5 mg./kg.-dose is used, the manufacturer recommends that a multiplication factor be used to compensate for the difference. Failure to use the factor will result in false positive results and liver dysfunction cases might even indicate more than 100 per cent retention, which would be impossible.

SUMMARY

1) A comparison was made between uric acid determination and sulfobromophthalein sodium (Bromsulphalein, BSP) retention results on a known liver condition.

2) Results indicate that the latter is much more sensitive than uric acid methods. When the uric acid level could be considered abnormal, BSP retentions were extremely high.

3) Heparin-lined syringes are recommended for blood withdrawals to reduce hemolysis.

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Experimental Distemper in Mink and Ferrets. II. Appearance and Significance of Histopathological Changes

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A METHOD of infecting mink and ferrets with distemper virus (DV) in a manner to simulate natural infection as nearly as practicable under experimental conditions was described earlier.[4] This report will describe the appearance of microscopic lesions in exposed animals and will also describe certain observations, apparently unrelated to disease, which could be confused with pathological changes.

Inclusion bodies of distemper were described independently by Standfuss[16] in 1908 and Lentz[9] in 1908 and 1909; both observed the inclusion bodies in the nerve cells of dogs with the neurological type of distemper. Sinigaglia[15] reported distemper inclusion bodies in the bronchial epithelium of a dog in 1912. Some investigators, viz. Dunkin and Laidlaw[6] and Goldberg and Volgenau,[7] did not report on such inclusions. The inclusion bodies are most frequently found in epithelial cells of the respiratory passages and in the transitional epithelial cells of the urinary system.

There is general agreement on appearance and location of the inclusion bodies of distemper. They are described as eosinophilic or acidophilic, rounded structures, varying greatly in size. Several refractile granules are frequently seen in the larger ones. Inclusion bodies of distemper occur much more frequently in the cytoplasm than in the nucleus. They have been seen in epithelial cells of the ureter, renal pelvis, skin, pancreas, salivary gland, stomach, colon, and adrenal gland. In contrast to those of infectious canine hepatitis (fox encephalitis), they do not occur in the nuclei of vascular endothelium or in hepatic cells.

A variety of stains has been used for the demonstration of distemper inclusion bodies. Green and Evans[8] tried hematoxylin and eosin, Giemsa's stain, Broadhurst's nigrosin, and Congo red. They found, as did Wisnicky and Wipf,[17] that hematoxylin and eosin gave the best results. Cordy[3] used Pollack's trichrome stain successfully. Shorr's S_3 stain,[14] introduced for staining vaginal smears, was adopted for the demonstration of distemper inclusions by Page and Green.[13] Angulo *et al.*[1] demonstrated inclusion bodies in the epithelial cells of mink bladders, using phase microscopy on unstained material.

Application of the fluorescent antibody technique to the study of distemper in dogs and ferrets has revealed new information relative to the nature of the inclusion bodies, pathological changes, and pathogenesis. Moulton and Brown[12] demonstrated that specific antibody labeled with fluorescein isocyanate became attached to inclusion bodies in the epithelial cells of the urinary bladders of dogs. Liu and Coffin[10] studied the pathogenesis of distemper in ferrets by the fluorescent antibody method. They detected viral antigen in cervical lymph nodes of ferrets two days after exposure by nasal inoculation. Soon after, they found viral antigen in mediastinal lymph nodes, mesenteric nodes, and spleen. They detected the antigen in respiratory, urinary, and cutaneous epithelium and in blood about one week later.

Other histopathological changes, described in relation to distemper, are of nonspecific types and may be due to secondary invasion, as indicated by the reports of Dunkin and Laidlaw[6] and Green and Evans,[8] who took pains to exclude other infectious agents from their experimental animals. Cordy[3] suspected that an interstitial pneumonia, which he observed in dogs with typical clinical distemper, was caused by DV, while the common bronchopneumonia was a result of secondary invasion.

De Monbreun[5] described pathological changes in dogs which had been sick for long periods of time. His description of nuclear inclusion bodies in reticuloendothelial cells and hepatic cells of a dog almost certainly indicates a mixed infectious hepatitis-distemper syndrome. He also described an interstitial pneumonia with numerous monocytes, which suggests secondary infection by bacteria.

The intensive discussion of distemper and fox encephalitis inclusions by Green and Evans[8] gives excellent descriptions of distemper inclusion bodies in mink and ferrets as well as in Canidae. They evidently examined dead animals only, and their descriptions apply to material obtained at the height of the disease and from animals infected naturally at unknown times.

Because this report presents the pathological picture at various phases of the dis-

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ease, the observations will vary in some details from those of other writers.

MATERIALS AND METHODS

The animals and virus used in these experiments have been described previously.[4] All mink and ferrets observed were exposed to Green's distemperoid strain of DV, selected because of its virulence for these species. Nearly all tissues were taken from animals freshly killed by exsanguination and immediately dropped into a 4 per cent solution of formaldehyde neutralized with calcium carbonate. A few specimens were taken from animals which had died an hour or less prior to dissection, or had died in outdoor cages during cold weather. Animals suspected of being decomposed were discarded. No appreciable postmortem decomposition was observed in the tissues that were studied, regardless of how the subject died or was killed.

Control tissues from ferrets were obtained from healthy, unexposed, young animals which were killed by exsanguination. Control tissues of mink were obtained from healthy young animals slaughtered for pelting. The fixed and dehydrated tissues were imbedded in paraffin and sections cut at a thickness of 7 μ. Duplicate sections of the tissues were stained with hematoxylin and eosin and with Shorr's S_3 stain, as recommended by Page and Green.[13]

Observations made of lesions in mink over a period of three years have been compiled in the accompanying descriptions. Changes of nonspecific nature are described when they can be regarded as part of the entire pathological evidence of distemper.

RESULTS

Certain features, which may be easily interpreted as pathological, were observed in tissues of both mink and ferrets. Because they were also found in tissues of healthy control animals, they are described here as an aid to future workers who may be limited by lack of controls. These features are: in the lungs, emphysema with clubbing of alveolar walls, atelectasis, congestion, mucoid exudate in bronchi, and multinucleated cells in the alveolar walls; in the cerebellum, both swelling and shrinking of the Purkinje cells, the latter resembling ischemic necrosis seen in human brains; in the spleen, pyknotic nuclei in many of the megakaryocytes and erythrocytes in the lymphatic nodules which created an impression of hemorrhage.

Significant lesions in tissues of mink and ferrets are reported in the following descriptions.

Microlesions Found in Tissues of Mink Exposed to Distemper Virus by Aerosol Inhalation.—Day 2 Postexposure (PE)—No significant lesions in any tissue examined, viz., lung, spleen, brain, bladder, kidney, small intestine, skeletal muscle, and liver.

Day 4 PE—Lungs—Three of four lungs were apparently normal. Numerous typical inclusion bodies were present in the bronchial epithelial cells of one lung. Spleen—Early evidence of necrosis was found, *i.e.*, several pyknotic nuclei in cells of lymphatic nodules. No significant lesions were observed in other organs.

Day 6 PE—Lung—Acute peribronchitis was present. In some lungs, inclusion bodies were numerous in bronchial epithelium; in others, they were scarce. Spleen—In lymphatic nodules, early indistinct inclusion bodies were seen; many cells had pyknotic or karyorrhetic nuclei. No significant lesions were detected in other organs.

Day 8 PE—Lung—Leukocytic debris was present in bronchi, and numerous prominent inclusion bodies were found in bronchial epithelium. Spleen—There were some karyorrhetic nuclei, fewer pyknotic nuclei than on PE day 6, and numerous inclusion bodies in lymphatic nodules. Liver—Several small inclusion bodies were found in epithelial cells of bile ducts. There were no significant lesions in other organs.

Day 10 PE—Lung—A mild, acute bronchitis was seen, with a few hemorrhages in alveoli and bronchi; there were numerous prominent inclusion bodies in bronchial epithelium. Spleen—Pyknotic nuclei were found in all parts of the spleen, but were present only in small numbers in lymphatic nodules. Small intestine—There were very few inclusion bodies in epithelial cells of villi. Liver—Numerous inclusion bodies were found in bile duct epithelial cells; some cells were vesiculated and ruptured. Bladder—Inclusion bodies varied from few to moderate in epithelium. Kidneys—No significant lesions were found.

Day 12 PE—Lung—Desquamation of epithelial cells into bronchial lumina was present; numerous inclusion bodies varied greatly in size. Spleen—There were numerous inclusion bodies in cells of lymphatic nodules, but necrosis was less than in tissues harvested on PE days 8 and 10. Small intestine—Several inclusion bodies were found in epithelial cells of villi. Liver—Moderate congestion was present in the parenchyma and numerous inclusion bodies

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were in the bile duct epithelium; cells were ruptured. Bladder—Variable numbers of inclusion bodies were found in different animals. Kidney—There was localized congestion in the parenchyma, with a few loci of inclusions in pelvic epithelial cells.

Day 16 PE—Lung—There were many small pneumonic areas; purulent exudate in bronchi and numerous inclusion bodies in bronchial epithelium were present. Spleen—Congestion, very little necrosis, and numerous inclusion bodies in lymphatic nodules were found. Liver—Numerous inclusion bodies were in the bile duct epithelium. Kidney—Some kidneys apparently were normal; others showed congestion in the parenchyma and numerous inclusion bodies in the pelvic epithelium. Bladder—There were many inclusion bodies in epithelial cells.

Day 18 PE (organs of a single mink)—Lung—Edema and many typical inclusion bodies in bronchial epithelial cells were present. Spleen—Congestion was seen, but no inclusion bodies were found. Liver—Moderate fatty degeneration of the parenchyma was present; there were also a few inclusion bodies in the epithelial cells of bile ducts. Bladder—Moderate concentration of large inclusion bodies was found.

Day 20 PE—Lung—Generalized interstitial pneumonia and much cellular debris in bronchi were evident. The number of inclusion bodies in the bronchial epithelium varied greatly between animals. Small intestine—Numerous intranuclear and some cytoplasmic inclusion bodies were found. Bladder—A few inclusion bodies were seen. Liver and kidney—No significant lesions were found.

Day 23 PE (organs of a single mink)—Lung—Inclusion bodies were found in the cells of all bronchi. There was less pneumonia, congestion, and bronchitis than on PE day 20. Small intestine—There were numerous intranuclear and some cytoplasmic inclusion bodies. Spleen—Lymphatic nodules were hypercellular; inclusion bodies were few, but present. Kidney and bladder—These were apparently normal.

Day 24 PE (organs of a single mink)—Lung—Bronchitis with much mucopurulent exudate was apparent; there were numerous inclusion bodies in the bronchial epithelium. Spleen—Inclusion bodies were rare and the tissue was almost normal. Bladder—A few inclusion bodies were found in one area. Kidney and small intestine—These apparently were normal.

Day 31 PE (organs of a single mink, exposed but never sick)—Lung, liver, kidney, bladder and small intestine—All appeared to be normal.

Day 32 PE (organs of a single mink)—Lung—Bronchitis with a moderate amount of mucopurulent debris in lumina. There were numerous inclusion bodies in bronchial epithelium. Spleen—Lymphatic nodules were hypercellular and sharply defined; nuclear inclusion bodies were numerous in lymphatic cells, but comparatively rare in cytoplasm. Small intestine—Numerous nuclear inclusion bodies were found in epithelial cells.

Day 36 PE (organs of a single mink)—Lung—Extreme congestion but no inclusions were seen. Kidney—Congestion was apparent, but no inclusions were found. Bladder—The bladder apparently was normal.

Other organs examined in this series, in which significant lesions were never observed, were skeletal muscle, brain, and pancreas and adrenal, thyroid, and salivary glands.

Microlesions Found in Tissues of Ferrets Exposed to Distemper Virus by Aerosol Inhalation.—A similar series of observations was made on ferret tissues. The ferrets were 10 to 12 weeks old. All observations were made during a single experiment, rather than over a period of years, as in mink.

Day 3 PE—The lungs, livers, kidneys, bladders, spleens, and small intestines apparently were normal.

Day 4 PE—Lungs—A few indistinct inclusion bodies were found in the bronchial epithelium. Bladders—A few small, indistinct inclusion bodies were present. Spleen—There were very small, indistinct inclusion bodies and much necrosis involving about one half of all cells in many nodules. Liver, kidney, and small intestines—No significant changes were apparent.

Day 6 PE—Lungs—Cellular debris and occasional hemorrhages were found in bronchial lumina; several inclusion bodies were found in the bronchial epithelium. Spleen—Necrosis and numerous inclusion bodies were evident in cells of lymphatic nodules. In all other organs there were no significant lesions.

Day 8 PE—Lungs—There was acute bron-

chitis with a purulent exudate in the lumina; numerous inclusion bodies were found in the bronchial epithelium. Liver—There were numerous inclusion bodies in epithelial cells of bile ducts. Kidney—Inclusion bodies were found in several cells of the pelvic epithelium. Bladder—Numerous inclusion bodies were present. Spleen—There were numerous inclusion bodies and a few necrotic cells in lymphatic nodules. Small intestine—Inclusion bodies were suspected in several cells, but were difficult to identify.

Day 10 PE—Lungs—Small bronchioles were filled with mucus and cellular debris; the bronchial epithelium generally was frayed because of swelling and desquamation of cells. The number of inclusion bodies varied between ferrets and also between areas within single lungs; there was much congestion, and bronchitis was evident. Liver—Epithelial cells of the bile ducts were swollen, some were ruptured, and they contained numerous inclusion bodies. Kidney—There was a small number of inclusion bodies in pelvic epithelium. Bladder—Several inclusion bodies were found in the epithelium. Spleen—Numerous inclusion bodies were present in the cells of the lymphatic nodules; necrosis of lymphatic nodules varied between animals; some were hypercellular. Small intestine—Inclusion bodies were numerous in 1 ferret and scarce in the other.

Day 12 PE—Lung—(organs of a single ferret)—There was much fraying in the epithelial cells of bronchi and a moderate amount of exudate. Numerous inclusion bodies were present. Liver—Numerous inclusion bodies were found in the epithelial cells of the bile ducts. Kidney—Small numbers of inclusion bodies appeared in the pelvic epithelium. Bladder—Irregularity and disintegration of epithelial cells was found, as well as several inclusion bodies. Spleen—Necrosis and moderate numbers of inclusion bodies were present in cells of lymphatic nodules. Small intestine—There was a moderate number of inclusion bodies in epithelial cells.

Perusal of the descriptions reveals comparatively little difference in the significant lesions appearing in the two species. Mink showed more ability than ferrets to regenerate lymphoid tissue in the spleen after the necrotic lesions had appeared and persisted for several days.

The necrosis of lymphatic cells was also seen in mediastinal lymph nodes of 3 ferrets; 2 of these were infected by aerosol inhalation and 1 by subcutaneous injection. The aerosol-infected ferrets were killed 13 and 14 days postexposure when sick. The ferret inoculated subcutaneously died 21 days after exposure. It had been sick one week.

The lymph nodes presented a striking appearance under the microscope. Many lymphocytes were necrotic; in some areas they appeared to have been removed, leaving vacancies occupied by reticular cells or lymph. The reticular cells were enlarged or swollen and many contained one or more inclusion bodies, although these were much less plentiful there than in the epithelial cells of bronchi bile ducts. Interspersed with the affected areas were variable-sized areas of apparently healthy lymph nodes.

The incubation period among aerosol infected animals, judged by the appearance of clinical signs of disease, was never less than eight days and often more than ten. Animals selected for necropsy after the first eight or ten days postexposure usually showed signs of distemper, *i.e.* anorexia, lethargy, photophobia, conjunctivitis, and bile-stained feces. These signs appeared in nearly all sick animals and often occurred almost simultaneously. Since active virus was detected in tissues harvested from animals in the second day of incubation, an infected animal may be regarded as a potential carrier for eight or more days before becoming ill.

Time and viral titer seem to have definite effects on the formation of inclusion bodies, but absolute correlations cannot be drawn from our data. The appearance of these inclusions in the cells of a tissue varied considerably from one tissue to another, but virus was always detected for an appreciable period before inclusion bodies became visible. In some cases a few faint, acidophilic bodies were found in a tissue four or six days postexposure. The same tissues, from an animal killed two days later, usually contained several well-developed inclusion bodies. Apparently, these appear first as poorly-formed, indistinct "ghosts" and later acquire a clearly defined, sharp-staining quality by which they are readily differentiated from other cell components. They also appear to increase in size with the passage of time.

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Virus was demonstrated in brain, skeletal muscle, and several glands, but no inclusions were found in those tissues.

DISCUSSION

The widespread occurrence of inclusion bodies of distemper agrees with the pantropic nature of the causal virus. The presence of active virus in tissues several days before the appearance of inclusions was a constant finding in these experiments and suggests a close relationship between the virus and the inclusion body. The findings of Moulton and Brown,[12] Liu and Coffin,[10] and Moulton[11] with fluorescent antibody indicate a high DV antigen content in the inclusion bodies. This would suggest the inclusion consists of virus particles or of virus particles plus some cellular component which holds them together.

The ability to necrotize cells has not ordinarily been assigned to distemper virus. In this work, considerable cell destruction was seen in lymphatic tissues, *i.e.* lymph nodes and lymphatic nodules of the spleen. Necrosis was of no apparent importance in other organs, even in animals found dead or killed *in extremis*. The possibility that lymphatic destruction is related to the lethal mechanism of distemper is therefore advanced. No clear explanation of the lethal mechanism of distemper has been made, to the authors' knowledge, although in animals in which extensive damage to nervous tissue has taken place the explanation seems obvious. In our experience, there was little evidence of brain damage clinically or on histopathological examination. Likewise, pneumonia was rarely, if ever, extensive enough to account for death. Dyspnea and cyanosis were not part of the clinical syndrome. The fact that mink seem able to repair the damage to the spleen while the more susceptible ferrets do not, may also indicate a relationship between necrosis of lymphatic tissue by DV and the lethal mechanism of the disease.

SUMMARY

The progress of pathological changes taking place in mink and ferrets, experimentally infected with distemper virus, was charted in relation to time and, in some organs, to viral titer of tissues.

Inclusion bodies were more abundant and larger in epithelium of bronchi and bronchioles than in other tissues. They were demonstrated in transitional epithelium of the urinary system, epithelium of the hepatic bile ducts, epithelium of the small intestine, and in reticular cells of the lymph nodes and lymphatic nodules of the spleen.

Inclusion bodies were not demonstrated in cells of the brain, muscle, thyroid, adrenal or salivary glands although virus was demonstrated in those organs at some time during the course of the disease. No tissue assayed for viral content was found to be totally resistant to invasion by distemper virus.

Necrosis was seen in lymphatic nodules of the spleens of mink and ferrets and in lymph nodes of ferrets; lymph nodes of mink were not sectioned. Necrosis was not seen in other tissues. The destruction of lymphatic tissue is suspected of playing a role in the lethal mechanism of distemper.

Virus was always demonstrable, in tissues assayed for viral content, before inclusion bodies could be demonstrated. The organs in which the most pronounced changes occurred were usually those with the highest viral titer and vice versa.

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Studies on a Disease in Mink with Systemic Proliferation of the Plasma Cells

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TUMORS arising from plasma cells in bone marrow have long been recognized in human beings. On the basis of his morphological studies, Von Rustizky [49] introduced the term, "multiple myeloma." The more adequate name, "plasmacytoma," has subsequently come into use.

Plasmacytomata in human beings may also have an extramedullary localization, usually in the upper respiratory tract or conjunctivae and, less commonly, in the lungs, stomach, intestines, etc. The tumors may be benign or may be malignant and metastasize.

In the rare plasma cell leukemia, plasma cells appear in the blood and as diffuse infiltrations in the bone marrow, spleen, liver, lymph nodes, and other organs.

In human beings, plasmacytomata which arise from bone marrow are more common than the other forms of tumor-like or systemic plasma cell proliferation. Such tumors are usually of multicentric origin, but may also appear primarily as a solitary lesion and subsequently become multiple. Skeletal pains are intense, and spontaneous fractures often occur. Men, 40 to 60 years old, are most frequently affected. Renal lesions and serious abnormalities in protein metabolism are accompanying features. Serum albumin is usually depressed and the globulin fractions are increased. Involvement of bone marrow may lead to severe anemia. Atypical amyloidosis is seen fairly often, with deposition within the tumors, in the skin, around the joints, and in the skeletal musculature, heart, and lungs.

REVIEW OF LITERATURE

Plasmacytomata Arising from Bone Marrow.—Geschickter and Copeland [18] reviewed the literature concerning 425 incidences of plasmacytomata arising from bone marrow and discussed 13 other cases. This review was a valuable contribution to knowledge of the clinical features of the disease in human beings. In most patients with plasmacytomata in the bone marrow, the characteristic renal lesions, "myeloma kidney" with albuminuria, Bence Jones proteinuria and, in the final stages, uremia appear. Eosinophilic casts, often laminated and occasionally reaching imposing proportions, are present in the convoluted tubules, Henle's loop, and in the collecting tubules.[7] According to Bell,[6] these casts cause atrophy of the nephrons with an increase in the interstitial connective tissue. Circular, hyaline droplets in the tubule epithelium which take a positive Weigert's fibrin stain have been described.[1, 6, 14] It has been suggested that these represent resorption of protein.[1, 41]

Many investigators have been unable to detect changes in the glomerulus,[14, 20, 30] while others have observed thickening of the basement membrane.[5, 6, 36] Batts [5] and Morrison [36] also found fibrosis in the capillary tufts and pericapsular fibrosis.

According to several investigators,[6, 7, 11, 14, 21] the casts cause intrarenal urine stagnation (hydronephrosis) and uremia. Randerath,[41] however, considers the renal lesion primarily a "Speicherungsnephrose" with glomerular damage arising from the protein excretion. According to Apitz,[1] the casts are formed by condensation of the protein in the tubules and secondarily cause hydronephrosis. Höpker [22] compared clinical observations and pathological lesions in four patients with multiple myeloma. The concentrating capacity of the kidneys was depressed, and uremia was present. Tubular casts could be found and, in one instance, a few glomeruli were fibroid. He concluded that the renal lesions did not cause the uremia, since only one fourth to one third of the nephrons were obstructed. Extrarenal factors were considered to have caused the disturbance in renal function.

The amyloid deposits which are associated with plasmacytomata in bone marrow usually do not take amyloid stains and have been called paramyloid. Paramyloidosis has been observed in about 6 per cent of 643 cases of myeloma,[4] and a frequency of 10 per cent has been reported for another series.[26] Typical cases of amyloidosis with deposits in the liver, spleen, and kidneys have not been observed in combination with plasmacytomata.[26]

Extramedullary Plasmacytomata.—Jackson *et al.*[23] described 5 cases of extramedullary plasmacytoma. In two of the patients, plasmacytomata subsequently appeared in the skeleton, and the writers concluded that there was no essential difference between extramedullary, benign plasmacytomata and malignant, multiple plasmacytomata in the bone marrow. It appears from Hellwig's [19] survey that, in approximately half of the plasma-

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cytoma patients with the primary lesions in the respiratory tract, metastasis can appear without any alteration in the histological appearance. Hellwig [19] maintains that plasmacytomata generally arise as local, benign lesions in mucous membranes which, after a long interval, may become generalized. Furthermore, it is difficult to determine whether it is a true metastasis or of a multicentric origin.

In Stout and Kenney's [46] series of 106 patients with plasmacytoma in the upper respiratory tract, metastases to the regional lymph nodes or bone marrow but not to other internal organs occurred in 54 of the patients. It was concluded that the lesions were primarily multiple, and that true metastasis had not occurred, since the lungs were not involved.

Plasma Cell Leukemia.—Aschoff [3] apparently described the first case of this rare form. The diagnosis was based on the detection of plasma cells in a postmortem blood sample from a patient with plasmacytoma. Only 36 cases appear to have been described up to 1951.[25] According to Moss and Ackerman,[27] death usually occurs within eight months after the first clinical signs appear. The leukocyte count is generally only moderately elevated (15,000–30,000 per cmm.)[22] The relative number of plasma cells varies from a few per cent to 80 or 90 per cent. Those cases in which plasma cells never, or only occasionally, appear in circulating blood have been termed aleukemic or subleukemic plasma cell leukemia.[24]

In a few patients, this form may be associated with hyperglobulinemia, Bence Jones proteinuria, and renal lesions similar to those occurring with plasmacytomata in bone marrow.[38, 45] These alterations, however, are generally absent.[44] Diffuse osteoporosis is generally found in the bone marrow;[40] however, the skeleton may be free from lesions.[25] In addition to the diffuse infiltrations of plasma cells, which are apparent only microscopically both in bone marrow and extramedullary tissues, tumorous collections of plasma cells are commonly present.[2, 29, 34, 38, 39, 45] These cells display various degrees of maturity. According to Apitz,[2] this form of the disease is probably more common than the number of published reports would suggest, but diagnosis may be difficult, especially in patients with immature cell types.

At the present time, there is a tendency to consider plasmacytomata in bone marrow, local extramedullary plasmacytomata, and plasma cell leukemia as various manifestations of one disease. Frequently, plasma cell leukemia may represent an intermediate stage between this form and plasmacytomata in bone marrow.[21, 30, 44] Plasma cell proliferation is not limited to the bone marrow and may occur in the liver and kidneys as well.[28] Several investigators have observed a diffuse increase of plasma cells in the bone marrow in patients with macroscopically well-demarcated plasmacytoma.[2, 18, 43, 47]

Macroscopic visceral lesions are usually absent in patients with plasmacytoma in the bone marrow. Lowenhaupt [27] histologically examined several organs from 12 patients and found plasma cells diffusely distributed throughout the bone marrow and in the lymph nodes in each instance, in the spleens of 11 patients, and in the livers of six. In her opinion,[27] the tumor should be considered potentially diffuse, although the original lesion is local. Gordon and Churg [16] found extramedullary plasma cell infiltrations involving mainly the spleens, livers, and lymph nodes in 22 of 30 patients (73%). Similar observations were made by Hayes *et al.*,[17] who found extramedullary changes in 27 of 38 patients (71%). Gordon and Churg [16] considered bone marrow lesions to be more frequent and constant, and believed that visceral involvement probably represented an extension of the bone marrow lesions. The stimulus which initiated plasma cell proliferation in the bone marrow may have affected the potential hematopoietic activity of other organs. Like the leukemias, this tumor has a multicentric origin, and lesions in various organs cannot be considered to be true metastases. A similar opinion is held by Hayes *et al.*[17]

Rubinstein [44] also believes that there is no sharp distinction between plasmacytomata in bone marrow and plasma cell leukemia, and that the former should be considered as leukemia of plasma cells. Newman *et al.*[38] came to a similar conclusion after examining a patient with diffuse plasma cell infiltration in bone marrow, tumorous plasma cell infiltrations in the skin, and diffuse infiltrations in most of the internal organs.

Normal human bone marrow contains up to 1.1 per cent plasma cells. An increased plasma cell count (greater than 3%) alone is insufficient for the diagnosis of plasmacytoma. In 30 patients with liver cirrhosis, Jarrold and Vilter [24] regularly found an increase of plasma cells in bone marrow, to 10.5 per cent on the average, and hyperglobulinemia. The plasmacytosis in these patients was considered caused by an antigen which the damaged liver either liberated or was unable to break down. Bone marrow plasmacytosis was observed by Fadem and McBirnie [12] in Hodgkin's disease, lymphosarcoma, acute monocytic leukemia, refractory primary anemia, hernia, and papilloma. The plasma cells were mature and often degenerated. Hayhoe and Smith [18] found 1.8 to 6.3 per cent plasma cells in the bone marrow and hyperglobulinemia in ten patients with rheumatic arthritis. The plasma cell proliferation and arthritic lesions were believed to have been caused by the same agent.

Clark and Muirhead [10] examined 727 bone marrow biopsies and found that 76 had 5.0 per cent or more plasma cells. Plasmacytoma was present in 1.8 per cent of the entire series. In 8.7 per cent of the patients, a diagnosis of benign plasmacytosis was made. In these patients, the plasma cell count, their appearance, and other clinical findings were taken as the basis for diagnosis. The plasma cells in the plasmacytosis patients were generally mature, but immature cells were also present. They could not be distinguished from those which occurred in plasmacytomata, and it was necessary to base the diagnosis on the other clinical features. Plasmacytosis was present in

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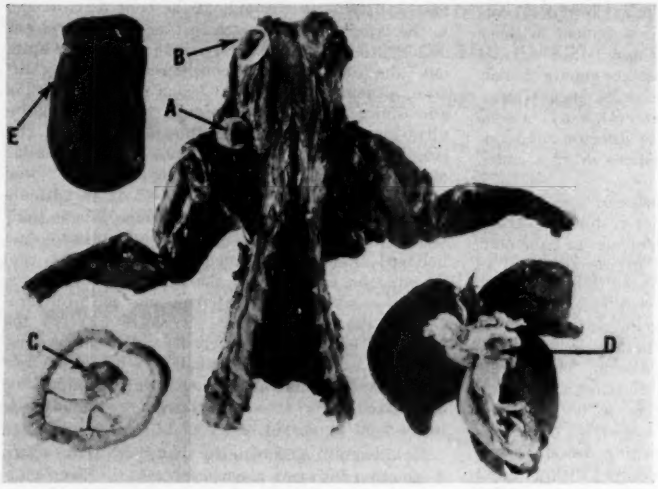

Fig. 1—Portion of a mink cadaver (thorax opened) demonstrating the lesions of systemic plasma cell proliferation. The prescapular (a), retropharyngeal (b), mesenteric (c), and portal (d) lymph nodes are moderately enlarged. Pronounced splenomegaly (e).

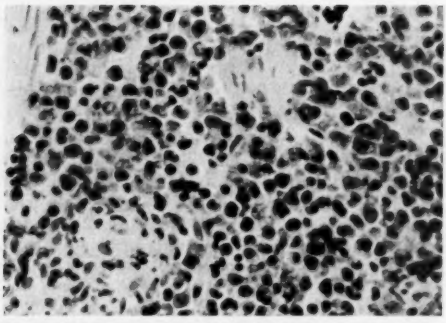

Fig. 2—Spleen of a mink. Immature plasma cells predominate and are scattered throughout the red pulp. H & E stain; x 333.

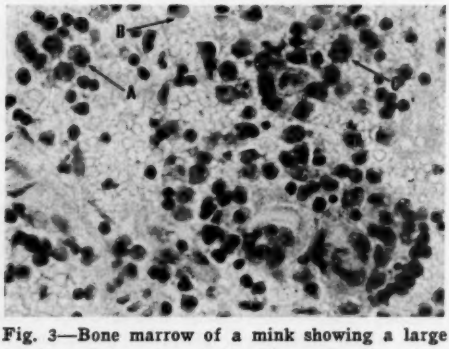

Fig. 3—Bone marrow of a mink showing a large number of immature (a) and occasional mature (b) plasma cells. H & E stain; x 333.

five major disease groups: (1) drug sensitivity; (2) collagen diseases; (3) chronic infections, espepecially granulomata; (4) liver cirrhosis; and (5) malignant tumors.

Numerous plasma cells were observed by Meurman [35] in the bone marrow, liver, spleen, and lymph nodes of a patient with liver cirrhosis and in the bone marrow of a patient with anemia, fibrinous valvular endocarditis, and chronic glomerulonephritis. A reactive plasmacytosis was considered to have occurred in these instances.

In animals, plasma cell tumors or leukemia are rare and have been reported in laboratory animals only. Bullock *et al.*[9] found a transplantable plasmacytoma which developed around a cysticercus in the liver of a rat. Plasma cell leukemia and plasmacytomata were observed by Rask-Nielsen and Gormsen [42] in a strain of mice. Unfortunately, the blood and bone marrow were not examined.

A disease of mink which appears to have some features in common with the disease to be described in the following sections of this paper has been described in the U.S.A. under the name, "Aleutian disease." [20]

Materials and Methods

This report deals with the pathological findings in 36 mink with a disease which appears to share many features with plasma cell leukemia in human beings. Six cases were encountered among a group of 34 clinically-normal Sapphire mink which were killed for pelting and originated from an establishment where many mink had been affected previously. The other mink had been submitted for routine necropsy. Of these 36 mink, 6 were Standards, 21 were Sapphires, 4 were Pastels, 1 a Blue Irish, and 1 an Aleutian. Three of the mink had been pelted and the color type could not be determined.

Tissues were fixed in 10 per cent formalin and

Carnoy's fluid. After they were embedded in paraffin, the Carnoy-fixed material was stained with methyl green pyronine by Kurnick's method and the formalin-fixed tissues were stained with hemalum-eosin, Giemsa's method, Gömöri's silver stain, azan, methyl violet, toluidine blue, and Hueck's iron stain.

RESULTS

The disease mainly affected mutation mink, but it was seen in phenotypical Standard mink. No clinical signs were seen in the early stages, but anemia, emaciation, and polydipsia appeared terminally.

Macroscopic Findings.—Mink which died spontaneously were generally in a poor state of nutrition. A moderate anemia was present in 7 mink. In approximately half of the affected mink, the body lymph nodes were moderately enlarged and were usually yellowish brown. A few mink had greatly enlarged lymph nodes, up to 8 by 3 mm. Splenomegaly was present in all except 2 mink, and the cut surfaces bulged, were greyish red, and lacked distinct follicles (fig. 1). In 3 mink, the spleens were greatly enlarged and weighed between 10 and 16 Gm. In typical cases, the bone marrow was a light, greyish red. The lung lymph nodes were often slightly enlarged and, occasionally, the portal and mesenteric

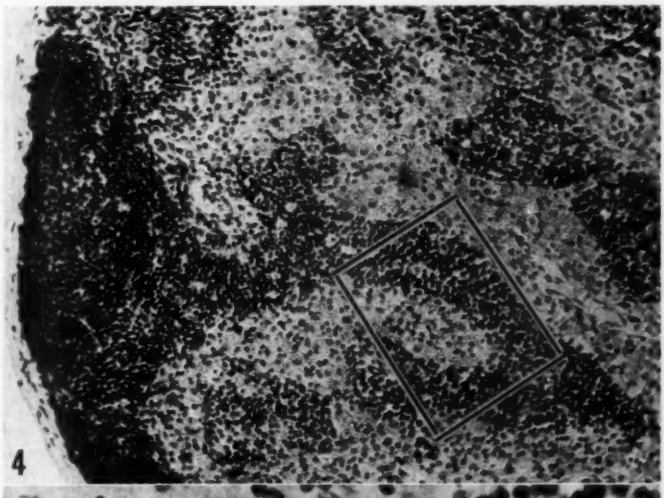

Fig. 4—Prescapular lymph node of a mink showing a large number of plasma cells in the medullary cords and in the cortex around atrophic follicles. H & E stain; x 150.

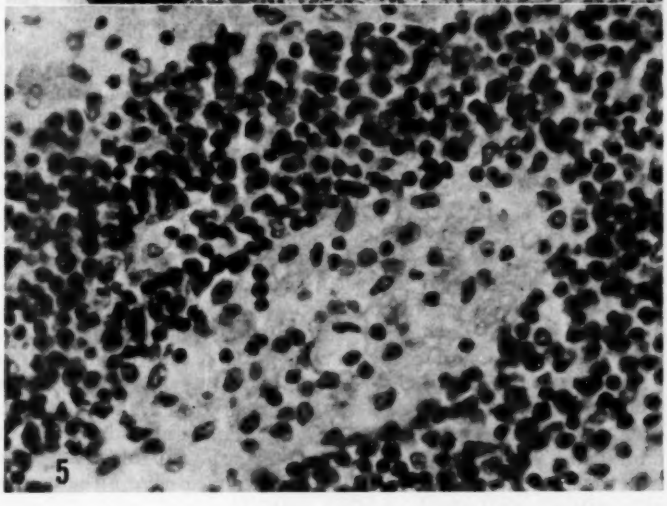

Fig. 5—A greater magnification of the blocked area in figure 4. Predominantly mature plasma cells with occasional immature forms. H & E stain; x 500.

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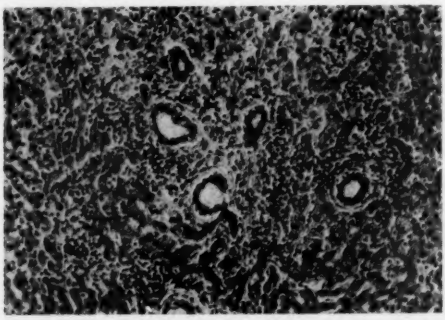

Fig. 6—Liver of a mink showing a large number of mature plasma cells periportally, smaller foci perisinusoidally, and moderate bile duct proliferation. H & E stain; x 100.

lymph nodes were moderately enlarged. In 5 mink, the liver had a light color (fatty change), and in 1 mink, diffuse liver necrosis and liver rupture were present. Hemorrhagic erosions in the gastric fundus region with blackening of the intestinal contents were observed in the mink which died spontaneously or were killed *in extremis*.

In 17 mink, the kidneys were somewhat enlarged, yellowish or brownish grey, and had a slightly to moderately increased consistency. Fine, dark flecks were thickly scattered throughout the cortex to give the surface and cut surface a blotched appearance. A strong odor of ammonia emanated from the gastric mucous membrane in 14 of these animals. The 3 mink in which uremia was not present in spite of macroscopical kidney lesions either had been killed or had died of intercurrent infection (1 mink was killed, 1 died of pasteurellosis, and 1 died of a ruptured spleen). No signs of uremia could be observed in the 6 clinically normal Sapphire mink which had been killed. Sufficient urine was present in the bladders of 5 cadavers for the determination of Bence Jones protein. In 4 of these animals, the heat-coagulability test for this protein was positive.

Bacteriological Examinations.—Since past experience did not suggest a relationship between pathogenic bacteria and this disease, bacteriological examinations were made on only a limited number of mink. The livers and spleens from 11 of the mink, however, were examined as a routine measure. No bacteria were found in 5 of the mink, and in 4, only coliform organisms were demonstrated. *Pasteurella multocida* and *beta*-hemolytic streptococci were each isolated on one occasion.

Microscopic Findings.—In all but 1 mink, large numbers of plasma cells were present in the spleens, distributed diffusely or as small foci in the red pulp. Both mature and immature cell types occurred, with the immature cells predominating (fig. 2). The immature form was relatively large and had a large, light, leptochromatic nucleus with one or several nucleoli and a weakly pyroninophilic cytoplasm. Megakaryocytes and erythropoietic foci were present in the red pulp, often to a great extent. The lymph follicles were few and, in many instances, atrophic. Pronounced hemosiderosis was present in most mink.

The bone marrow in the 27 mink examined contained an increased number of plasma cells. This increase was slight to moderate in 4 animals, and quite pronounced in the remaining mink. As in the spleen, immature types predominated (fig. 3).

The lymph nodes invariably contained large numbers of plasma cells in the medullary cords and often in the cortex around the follicles as well (fig. 4). Mature cells predominated, but large numbers of immature cells were occasionally seen (fig. 5). In a number of animals with immature plasma cells, polynucleated giant cells were also present. The lymph follicles were often atrophic (fig. 4). Hemosiderosis was pronounced in some cases. Six of the 28 mesenteric lymph nodes examined were normal, and the remainder showed increased numbers of plasma cells.

Small collections of plasma cells were present in 1 of 7 animals in which the laryngeal tonsils were examined. No increase in plasma cells in the tonsils was observed in the other animals.

A large number of plasma cells was present in the pulmonary lymph nodes in most animals. In the lungs of approximately half the animals, slight to moderate peribronchial and perivascular infiltrations of predominantly mature cells were seen.

In the livers of all but 1 animal, large and small collections of largely mature plasma cells could be found in the periportal tissues. Lymphocytes were often present as well. Foci of plasma cells occurred perisinusoidally in the more severly affected animals. Moderate proliferation of bile ducts in the periportal region could be seen

in 9 of the animals (fig. 6). Protein casts were often present in the proliferated bile ducts. Small fat droplets occurred in the cytoplasm of the liver cells in a few instances; fatty changes were present in the hearts and kidneys of these animals as well. In those animals in which hemosiderosis was present in the spleen, hemosiderin deposits were also seen in the Kupffer cells.

Slight plasma cell infiltrations were present in the gastric and intestinal mucous membrane in a few instances. There were 3 animals with chronic fibrous gastritis.

Focal plasma cell infiltrations were seen in the kidneys of 29 animals. These infiltrations may have been large enough to separate the convoluted tubules. Mature plasma cells predominated (fig. 7). There were two instances of glomerular changes resembling the "wire-loop" glomeruli seen in human beings (fig. 8). The homogenous deposits around the capillary walls did not stain metachromatically with methyl violet. The glomerular tufts in 2 animals were fibrotic. The glomerular space was dilated in several animals and contained homogenous, protein-like material. Hematuria was present in 2 animals, with escape of erythrocytes into the glomerular space and the lumina of the associated tubules (fig. 9). Homogenous or granular protein casts which take a yellowish brown color with Van Gieson's stain were present in the convoluted tubules, Henle's loops, and collecting tubules in 24 animals (fig. 7). Fatty changes in the renal epithelium were associated with fat droplets within the casts. The portions of the nephrons which were proximal to the casts were atrophic with an increase in interstitial connective tissue. In kidneys in which the casts were abundant, the connective tissue proliferation resulted in an appearance resembling that of chronic interstitial nephritis (fig. 10). Calcified casts were occasionally encountered in the medulla.

The testicles in 6 of 8, and ovaries in 11 of 14 mink contained slight, diffuse infiltrations of mature plasma cells. In 7 of 13 mink examined, plasma cells were present in the propria of the uterus and often in the other layers also (fig. 12).

Slight to moderate infiltration of predominantly mature plasma cells was present in the choroid plexuses of 12 of the 25 brains examined. In addition, 2 of the animals showed pronounced plasma cell infiltration of the pia mater.

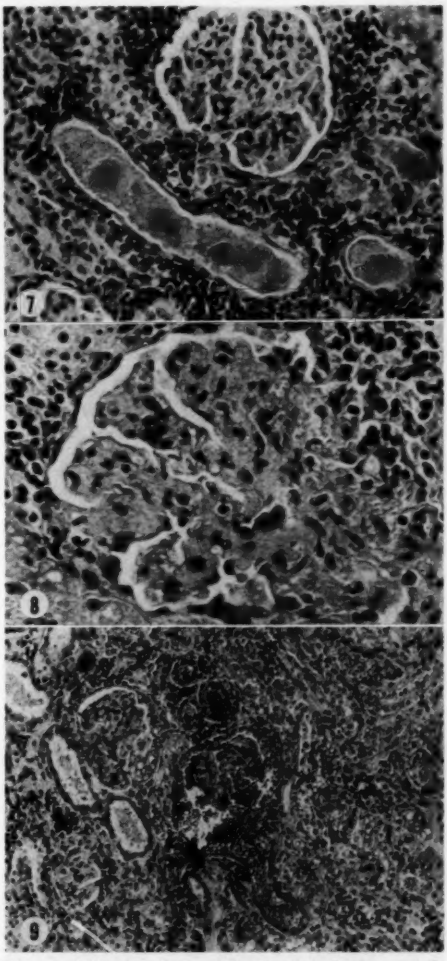

Fig. 7—Kidney of a mink showing large protein casts in the convoluted tubules. A large number of plasma cells are present in the interstitium and separate the tubules. H & E stain; x 200.

Fig. 8—Kidney of a mink showing homogenous deposits in the walls of the glomerular capillaries (wire-loop glomeruli). A large number of mature plasma cells are present in the interstitium. Van Gieson's stain; x 333.

Fig. 9—Kidney of a mink. The dilated glomerular space and associated tubules contain erythrocytes. H & E stain; x 100.

In several animals, the plasma cells in the bone marrow and viscera contained Russell's bodies (fig. 13).

Deposits of paramyloid were present in 15 mink (42%). These deposits were usu-

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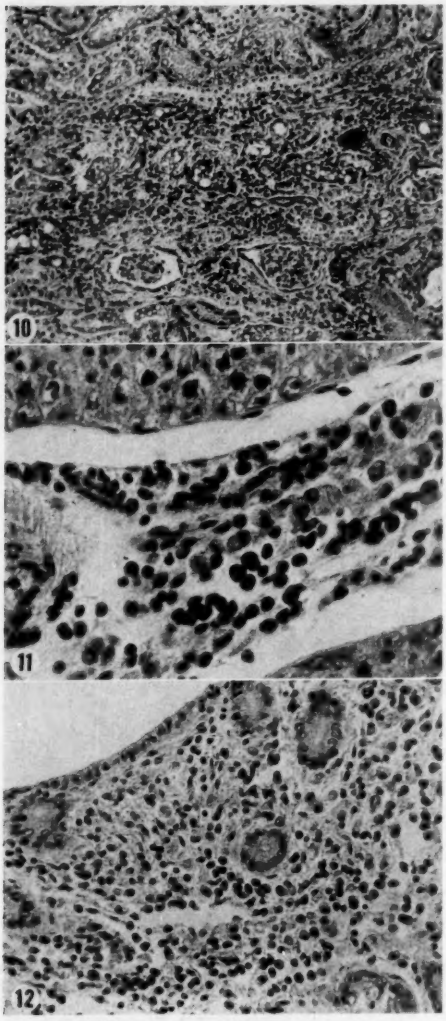


Fig. 10—Kidney of a mink showing a group of atrophic nephrons occupying the center of the field and a slight increase of interstitial connective tissue. H & E stain; x 100.

Fig. 11—Testicle of a mink showing slight plasma cell infiltration in the interstitium. H & E stain; x 333.

Fig. 12—Uterus of a mink showing diffuse plasma cell infiltration of the mucosa. H & E stain; x 333.

ally relatively small, and generally occurred in one site only. No metachromasia was observed with methyl violet. The body lymph nodes were involved in 5 animals, the pulmonary lymph nodes in 3, and the mesenteric lymph nodes in 2 (fig. 14). Paramyloid occurred either as deposits surrounded by fibroblasts or within the walls of the smaller vessels. A focal deposit of paramyloid surrounded by fibroblasts and plasma cells was seen in the lung parenchyma of 1 mink (fig. 15). Deposits in the walls of renal blood vessels were present in 4 animals. In 1 animal, a single deposit surrounded by fibroblasts and resembling the deposits in the lymph nodes was seen in the renal cortex.

Paramyloid could be seen in the spleens of 5 mink, in the follicles of 3 and in the red pulp of 2. Paramyloid deposits were present diffusely throughout the bone marrow of 1 mink, in the walls of the blood vessels in the bone marrow of 1, in the periportal connective tissue of 1, and in the submucosal connective tissue of the intestine of 1 mink.

Typical amyloidosis was present in 2 animals with abundant perisinusoidal deposits in the liver and in the splenic follicles. These deposits were metachromatic with toluidine blue and methyl violet.

DISCUSSION

The pathological findings in the mink examined differ from those seen in plasmacytomata in bone marrow of human beings, mainly through the absence of tumorous collections of plasma cells. Morphologically, the mink disease is more uniform. Judging from the clinically normal mink killed for pelting, the disease begins with plasma cell proliferation in the bone marrow, spleen, and lymph nodes. Infiltrations appear in the liver and kidneys and then, in the final stages, infiltration of practically all organs occurs. These infiltrations never become macroscopically visible. Enlargement of the spleen and body lymph nodes is generally only moderate, and all lymph nodes are uniformly involved. Unlike the lymphatic leukoses of other animal species, distinct tumors do not appear in the lymph nodes or spleen with some lymph nodes being severely involved while others are not affected.

As the disease in mink follows its natural course, abnormalities appear which resemble those seen in plasmacytomata in bone marrow of human beings. Signs of disturbed protein metabolism can be seen in paramyloid deposits, occurrence of Bence Jones proteinuria, and renal lesions. Involvement of the bone marrow affects hemo-

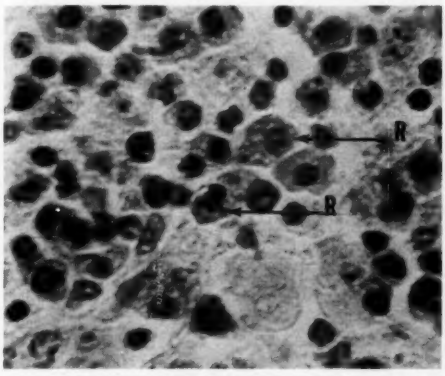
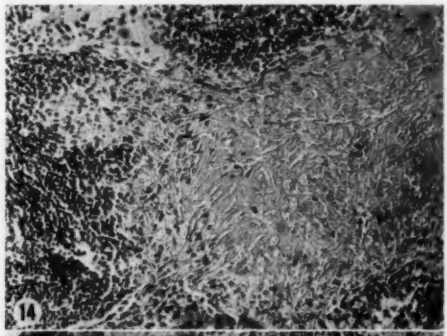


Fig. 13—Internal iliac lymph node in a mink. Some of the plasma cells in the medullary cords contain Russell bodies (R). Giemsa's stain; x 900.

poiesis and gives rise to anemia. As in human beings,[26,31] depression of erythropoiesis in mink is associated with hemosiderosis in liver, spleen, body lymph nodes, and bone marrow. Focal extramedullary erythropoiesis is often seen in affected mink and in human beings; the phenomenon has been observed in the liver [13] and in the liver and spleen.[8] Generally, there are no abnormalities in protein metabolism in plasma cell leukemia in human beings.[34,46] In other respects, the pathological findings in the mink closely agree with those seen in plasma cell leukemia in human beings. Because of the difficulties of obtaining clinical material, it was impossible to determine whether or not plasma cells were present in the circulating blood of the mink.

Plasmacytomata in bone marrow and plasma cell leukemia can be considered a form of leukosis. It may be difficult to distinguish reactive plasmacytosis from these, since immature cell forms appear in both instances.[10] The cytology of a plasmacytoma does not give any guide to the degree of malignancy.[17] The clinical courses of plasmacytomata and plasmacytosis are quite different, since the former are invariably fatal and the latter, a secondary phenomenon, generally have a satisfactory outcome. If death does occur in cases of plasmacytosis, it is the result of the primary disease. Hyperglobulinemia has been detected in cases of plasmacytosis,[10,18,24,35] but never paramyloidosis, Bence Jones proteinuria, or "myeloma kidney."

Neither in the mink described here nor



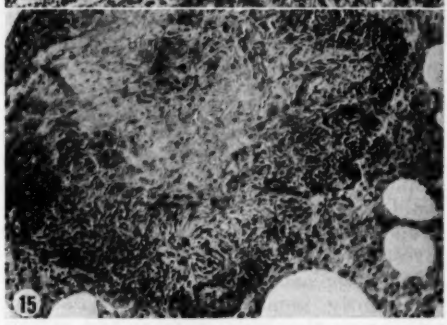


Fig. 14—Retropharyngeal lymph node of a mink showing a diffuse paramyloid deposit. H & E stain; x 100.

Fig. 15—Lung of a mink showing a focal subpleural deposit of paramyloid. Fibroblasts and a peripheral ring of plasma cells surround the deposit. H & E stain; x 100.

in other mink affected with the disease have bacteria been regularly isolated which could have given a reactive plasmacytosis. Distemper, as well as the lesions which in human beings give rise to plasmacytosis, have been eliminated as possible exciting factors. Since tumorous collections of plasma cells were not observed in any of the mink, it is impossible to determine solely on the basis of the plasma cell infiltrations whether these cases should be classified as plasma cell leukemia or plasmacytosis. Absence of any detectable primary disease, the presence of the characteristic renal lesions associated with plasmacytomata in bone marrow, as well as signs of other abnormalities in protein metabolism, and the fatal clinical course suggest that this mink disease should be considered a plasma cell leukemia. Experimental studies are being made and will include the examination of blood and bone marrow from living animals. Results of

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these investigations may assist in clarifying the question of classification of this disease of mink.

SUMMARY

A disease with systemic proliferation of plasma cells was described in 36 mink. The disease mainly affected mutation mink; 21 were Sapphire mink.

Clinical signs (emaciation, anemia, polydipsia) were evident only in the terminal stages.

Moderately enlarged lymph nodes were seen in half of the cases. Splenomegaly was almost invariably present, and the pulp was greyish red. Enlarged yellowish grey kidneys were often observed. Dark flecks were scattered throughout the cortex and the consistency was increased. Uremia had occurred in most animals with macroscopical renal lesions. Bence Jones protein could be demonstrated by the heat-coagulability test in four of the five urine samples investigated.

Microscopically, large numbers of predominantly immature plasma cells were present in the bone marrow and spleen. Large numbers of megakaryocytes and focal hematopoiesis were often seen in the spleen. Periportal and, on occasion, perisinusoidal collections of predominantly mature plasma cells were present in the livers. Moderate bile duct proliferation was also seen in nine livers. A number of largely mature plasma cells was present in the body and pulmonary lymph nodes. Peribronchial and perivascular plasma cell infiltration occurred in the lungs of half the animals and, occasionally, infiltration of the gastric and intestinal mucous membrane was present. Slight infiltration of plasma cells was occasionally observed in the testicles, ovaries, uterus, and the choroid plexuses of the brain. Focal, often extensive, plasma cell infiltrations were present in the kidneys of 29 mink. Glomerular fibrosis was seen in 2 mink, and there were 2 animals with wire-loop glomeruli. In 24 animals, homogenous or granular protein casts were found in the convoluted tubules, Henle's loops, and collecting tubules. The portions of the nephrons proximal to the casts were atrophic, and focal connective-tissue proliferation had occurred.

Small paramyloid deposits were found in 15 mink and involved the lymph nodes, kidneys, spleen, bone marrow, liver, and lungs. Typical amyloidosis was seen in 2 mink, with abundant deposits in the liver and spleen.

The findings in mink are considered in relation to the changes in plasmacytomata in bone marrow, plasma cell leukemia, and reactive plasmacytosis in human beings. It is concluded that the mink disease described shows striking morphological similarities with plasma cell leukemia but that further investigations are necessary to resolve the problem.

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An Erysipelas Serum-Culture Agglutination (ESCA) Test

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APPEARANCE OF swine erysipelas in North America presented the problem of clinically recognizing a new disease in suspected cases and confirming the diagnosis by isolation and identification of the causative agent. To facilitate identification of the organisms of swine erysipelas, Breed [2] used a precipitation test based on tests described by Vanney and Ascoli.[12] Since then, the biochemical properties, culture characteristics, and resistance of *Erysipelothrix rhusiopathiae* have been reported.[1, 6, 8, 9]

Later, a laboratory tube test [10] and a whole blood, rapid agglutination test [4] for the diagnosis of swine erysipelas were described. These tests did not produce reliable results for Karlson and McNutt,[5] who preferred the use of a microscopic agglutination test.

All of the above tests were found to be erratic and unreliable by Brudnjak and Kralj [3] and by Wellmann.[13] Wellmann described a macroscopic precipitation test consisting of a series of test tubes containing horse meat infusion and varying amounts of blood serum of the suspected animal to be tested. The broth was inoculated with *Ery. rhusiopathiae* organisms to obtain growth in all the tubes. Significance of the test was the precipitation of organisms by antibody present at a serum dilution of 7.5 per cent or less. The author recognized shortcomings in his test and recommended that a hemagglutination-inhibition test also be conducted simultaneously.

It was pointed out by Schoening and Creech [11] and by Kelser and Schoening [7] that serological tests can not be used with sufficient reliability to make a diagnosis of swine erysipelas and differentiate it from several other diseases. Other authors called attention to the various uncontrolled factors influencing the results of these tests.[3, 5, 13]

With some modifications of the Wellmann precipitation test, specific circulating antibody to *Ery. rhusiopathiae* can be demonstrated and correlated to challenge resistance. The presence or absence of the antibody, however, is not necessarily of diagnostic import.

MATERIALS AND METHODS

Blood of animals to be tested was withdrawn aseptically, and the serums were inactivated at 57 C. for 30 minutes. Following inactivation, the serum samples were either used immediately or frozen for later use. It was necessary that the samples contain no bacterial contaminants and, if the latter persisted after inactivation, the serums were sterilized by filtration. Susceptibility studies required a minimum of 0.6 ml. of serum, but it was advisable to have 2.5 ml. of each serum for verification tests.

Erysipelothrix rhusiopathiae, strain XA2,* was restored from the desiccated state and transferred serially in tryptose broth three times. Each transfer was incubated for 24 hours after a loopful of the earlier culture was inoculated into the tubes containing tryptose broth. The third XA2 transfer was the seed for the erysipelas serum-culture agglutination (ESCA) test.

If held under refrigeration, the material was satisfactory for 5 to 7 days, but more reliable results were obtained by using fresh cultures. The seed, after the third transfer in broth, could be cultivated on tryptose agar slants for 24 hours and then refrigerated. The material remained satisfactory for seed preparation for approximately two months. During this time, a small amount of the growth was periodically removed with a loop, inoculated into broth tubes, incubated for 24 hours, and used as seed.

The ESCA test was based on adding the serums of animals to be tested to trytose broth in approximately 5 per cent and 1 per cent concentrations for susceptibility studies, and in smaller amounts for other tests of hyperimmune animals. The test was performed by placing 9.9 ml. of tryptose broth in a tube and 9.5 ml. in a second tube. The first sample required 0.1 ml. of serum and the second sample, 0.5 ml. The serum-culture mediums were then inoculated with a loopful of seed, the materials were mixed, and incubated at 37 C. for 18 hours.

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* Derived from culture C-1, supplied by Dr. J. Crawley, Connaught Medical Research Laboratories, Toronto, Canada.

As *Ery. rhusiopathiae* strain XA2 grew in tryptose broth, a finely dispersed, iridescent turbidity was produced. Similar growth was obtained in mediums with small amounts of serum, but the turbidity was heavier. Tubes showing irregularities were examined microscopically for purity. In some instances the organisms grew slowly, and additional incubation to 20 or 24 hours was necessary. The optimal incubation was best determined by examining the control tubes of known positive and negative serums.

Evaluations of ESCA patterns required history surveys and challenge correlations of animal donors; for this purpose, three trials were conducted. The first trial involved 10 swine from a farm believed to be free of swine erysipelas. The animals were brought to the laboratory, placed in an isolation area, and blood samples were taken from the anterior vena cava. The animals were challenged with a mixture of various strains known to cause the disease. The skin challenge areas were clipped free of hair, cleansed, scarified with a sharp instrument, and moistened with a mixture of *Ery. rhusiopathiae* organisms. The first trial was used to evaluate ESCA reactions of probably susceptible animals.

A second trial involved 19 swine that were exposed to swine erysipelas with an experimental substandard bacterin. The animals were selected as being probably susceptible before exposure and, 21 days after the bacterin inoculations, they were challenged by the procedure described for the first trial. It was hoped that these animals would develop only low levels of immunity while they remained free of infectious agents.

A third trial consisted of evaluating serums of horses. Blood samples were drawn from the jugular veins of 12 horses and the serums were prepared as described previously. Ten of the horses were procured from farms and probably carried no *Ery. rhusiopathiae* antibody, and the other 2 were used for the manufacture of commercial anti-swine erysipelas serum. The serums were ESCA tested in the following dilutions: 1:20, 1:100, 1:1,000, 1:10,000, and 1:100,000. The third trial had two objectives: first, to study ESCA procedures using serums from species other than swine; second, to see how serums of hyperimmunized animals affected the ESCA test.

Most of the serums from trials 1, 2, 3, and the serums of chickens, dogs, and other animals were ESCA tested more than once. The various serums were coded and the results were compared.

RESULTS

Initial experiments included observations on the reactions of various *Ery. rhusiopathiae* strains to growth in the presence of suspected positive and negative serums. The identities of the organisms and serums were withheld, and the samples were presented to various bacteriologists for their observations. Generally, the reactions were recorded as cloudy growth, clumpy growth, or precipitated growth. After numerous recordings from 16- to 48-hour incubations, it was found that all strains produced consistent patterns with low and high concentrations of test serums. Also, there were many inconsistencies in the reactions and interpretations when intermediate amounts of serum were used.

TABLE 1—Relationship Between ESCA Tests and Challenge of 10 Swine

Animal No.	ESCA test: Serum concentration 5%	ESCA test: Serum concentration 1%	ESCA test: Interpretation*	Challenge reaction: Skin	Challenge reaction: Fever
9211	−	−	S	+	+
9233	−	−	S	+	+
9246	−	−	S	+	+
9261	+	±	P	+	−
9259	+	+	I	−	−
9279	+	+	I	−	−
9289	−	−	S	+	+
9282	−	−	S	+	+
9290	+	±	P	−	−
9314	−	−	S	+	+

* S = susceptible; P = partially immune; I = immune. Plus signs under ESCA indicate agglutination; plus signs under Challenge indicate reaction.

Of eight strains tested, only one produced consistent patterns regardless of the serum concentrations. This strain, XA2, was more resistant to agglutination with negative serum than the other strains, and nonagglutinated iridescent growth occurred with serum concentrations of up to 6 per cent of the total culture volume. Further efforts to standardize the reactions of XA2 were made with many serums, and the optimal time for reading ESCA was found to be after approximately 18 hours of incubation at 37 C. Three basic patterns were exhibited by the XA2 strain in the presence of various negative and positive serums: gross agglutinated growth, suspended agglutinated growth, and growth with no agglutination. The patterns were affected by the amounts of serums in the cultures, and it was always possible to dilute agglutinating serums to a point where they cause no agglutination.

Once the ESCA procedures were established, it was necessary to find the relationship between the growth patterns and the effect of actual challenge of animals which had served as serum donors. In the first trial, using 10 normal swine, it was found that serum from animals susceptible to

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TABLE 2—Relationships Between ESCA Tests and Erysipelas-Exposed Swine

Animal No.	ESCA test: Serum concentration 5%	1%	Interpretation*	Challenge reaction: Skin	Fever
95	+	+	I	−	−
9527	+	+	I	−	−
9529	+	−	P	+	+
9539	+	−	P	+	+
9531	+	+	I	−	−
9532	+	−	P	+	+
9533	+	+	I	+**	−
9534	+	−	P	−	−
9536	+	+	I	−	−
9547	+	−	P	+	+
9548	−	−	S	+	+
9549	±	−	LP	−	−
9550	+	−	P	−	−
9551	−	−	S	−	−
9552	±	−	LP	±	+
9553	+	−	P	−	−
9554	±	−	LP	+	+
9555	±	−	LP	−	−
9556	+	+	I	−	−

* S = susceptible; LP = low partial; P = partial; I = immune. ** Slight reaction.

Plus signs under ESCA indicate agglutination (± = partial agglutination); plus signs under Challenge indicate reaction.

scarification challenge caused no agglutination. Serums from animals which resisted challenge caused agglutination even when used in 1 per cent concentration. Serum from animals 9261 and 9290 caused agglutination at 5 per cent, but not at 1 per cent concentrations. One of these animals underwent an abortive infection, but the second showed no signs of illness (table 1).

Of the 19 ESCA-susceptible swine given the poor antigen in the second trial, 11 developed low titers and were designated as "partials" or "low partials;" 2 developed no detectable ESCA antibody and were designated as "susceptibles;" and 6 produced enough antibody to cause agglutination in both the 5 per cent and 1 per cent concentration (table 2). The latter animals were designated as "immune." Scarification challenge of the animals revealed a pattern similar to the one seen in the first trial, but there were two inconsistencies between the ESCA results and the challenge reactions (animals 9533 and 9551). The immune animal which showed the skin reaction recovered quickly, and it was not determined whether the transient lesion was caused by erysipelas or by secondary invaders. The other animal, 9551, did not become sick.

TABLE 3—ESCA Patterns of Some Horse Serums

Animal No.	History*	Serum dilutions ** 1:20	1:100	1:1,000	1:10,000	1:100,000
HS 1	F	−	−	−	−	−
HS 2	F	−	−	−	−	−
HS 3	F	+	−	−	−	−
HS 4	F	+	−	−	−	−
HS 5	F	−	−	−	−	−
HS 6	F	−	−	−	−	−
HS 7	F	−	−	−	−	−
HS 8	F	−	−	−	−	−
HS 9	F	−	−	−	−	−
HS 10	F	−	−	−	−	−
HS 106	HY	+	+	+	+	−
HS 110	HY	+	+	+	+	+

* F = farm horse; HY = hyperimmunized horse.
** Plus signs indicate agglutination.

Almost 300 swine were compared by ESCA versus challenge, and there was approximately a 90 per cent correlation between ESCA patterns and challenge reactions in immune and susceptible animals. Approximately 50 per cent of the animals with low levels of circulatory antibody, classified as ESCA partials, show challenge reactions of varied severity.

Serums from 8 of the 10 farm horses showed no agglutination in any of the concentrations, and serum from the other 2 showed agglutination only at 5 per cent concentrations. Serums from horses used for commercial purposes caused agglutinations even at dilutions of 1:10,000 and 1:100,000 (table 3). The farm horses purchased for production of anti-swine erysipelas serum were tested at various times, and their serums revealed a continual rise in antibody concentration as their hyperimmunization schedule progressed.

Serums of some chickens, cows, and sheep showed nonspecific *Ery. rhusiopathiae* agglutination. No agglutination was detected using serums of a few dogs.

Many of the samples were retested after freezer storage and, in all instances, the results correlated with prior results, though there was an indication of minor losses in antibody activity.

DISCUSSION AND CONCLUSIONS

A review of available serological procedures for *Ery. rhusiopathiae* indicates that agglutination and precipitation techniques developed were found to be unreliable by some workers. Because of a need for a serological test to aid in swine erysipelas studies, it was necessary to evaluate some of the tests and modify them, if necessary, to obtain reliable indications of antibody.

The procedure described by Karlson and McNutt [5] was found to be reliable in detect-

ing negative and strongly positive serums, but proper interpretation of the test usually required familiarity with the agglutination patterns of the organisms used, as well as comparisons with control samples. When serums of low antibody concentrations were used, it was difficult to interpret the test.

The ESCA test, a culture-agglutination test similar to one described by Wellmann,[13] was found to be reliable and easy to interpret. Efforts were made to use only easily available materials and standardized procedures so that constant patterns might be achieved by anyone using the test. Many strains function in ESCA tests, but strain XA2 appears to be more resistant to agglutination with negative serums and gives patterns that can be replicated.

The ESCA test described in this report can be used to separate swine into three groups, susceptible, partially immune, and immune, depending upon their resistance to scarification challenge. Because of the abortive nature of the disease in partially immune animals, it is thought that they would resist infection under normal conditions. Theoretically, ESCA failures within immune groups might be due to nonspecific antibodies stimulated by the other antigens and in the susceptible group might be the result of strain multivalency of *Ery. rhusiopathiae* or lack of circulating antibody. Preliminary studies on serums of various species have shown cross reactions between *Ery. rhusiopathiae* and other organisms. These findings will be reported elsewhere.

There are some shortcomings of the ESCA test; sterile serums are necessary, evaluations of samples take at least one day, and it is impractical to set up the test in the field. However, the test has proven useful in some assays of immunity, and it has proven to be reliable.

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XUM

Isolation and Propagation of Bluetongue Virus in Tissue Culture

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DURING THE SUMMER of 1956, an outbreak of catarrhal fever of sheep (bluetongue) occurred in Portugal.[6] The high morbidity and mortality of this disease, which appeared that year for the first time in Europe, placed it among the most important diseases of sheep in that country.[16] Studies on the propagation of bluetongue virus (BTV) in tissue culture with the object of developing a serum-virus neutralization and complement-fixation test were made in Onderstepoort by Haig *et al.*[8] and Shone *et al.*[15] Attempts to cultivate this virus *in vitro* in order to show its presence by induction of cytological damage were made in 1956 in Lisbon.[13] The present paper deals with the successful isolation and propagation of bluetongue virus in various tissue culture sytems. Studies on the virus-serum neutralization test were made in order to show the possible use of this phenomenon for diagnostic purposes in tissue culture.

MATERIALS AND METHODS

Virus.—Two strains of BTV were used. One was egg-adapted and was procured from the Texas Agricultural Experiment Station (specimen 119, 1955). This virus was used at the thirty-sixth passage level. The other was obtained from the blood of a sheep in the acute phase of the disease and isolated in trypsinized lamb kidney cells. The viruses were stored at 4 C.

Serum.—Convalescent serum was obtained from sheep that had recovered from experimental infection. The serum was stored at −20 C.

Tissue Culture Procedures.—Lamb kidney cells were prepared by trypsinization of the cortices of a newborn lamb's kidneys, employing with some modifications the techniques of Dulbecco and Vogt[4] and Youngner.[18] The cortices of both kidneys were cut into fragments approximately 2 mm. square. These were washed several times with Gey's balanced salt solution (BSS). The fragments were then cut into small pieces with iridectomy scissors. They were washed three times in BSS, containing 1,000 units of penicillin and 1 mg. of neomycin per milliliter, and placed in a 250-ml. Erlenmeyer flask. For digestion, 30 ml. of 0.25 per cent trypsin in Gey's BSS without calcium and magnesium, which had been preheated to 37 C., was added to the flask containing the kidney fragments and a small magnetized iron cylinder which was coated with neutral glass. The flask was then placed on a magnetic mixer and the fragments were stirred at a slow rate for 25 minutes. After removal from the mixer, the flask was allowed to stand for about one minute before the supernatant fluid was removed and the same procedure was repeated. The fluid from the subsequent trypsinization procedures was collected in 40-ml. centrifuge tubes and placed in the refrigerator until the entire process was completed. The cells were then centrifuged at 1,000 r.p.m. for 15 minutes at approximately 4 C. The trypsin solution was decanted and discarded. The cells were washed twice in Eagle's medium, centrifuged again for 15 minutes at the same speed, and finally resuspended in Eagle's medium. They were then counted by means of a hemocytometer and dispensed in roller tubes and flasks (10^6 cells in 1 ml.); these were incubated at 37 C.

The following strains of cells were used: human amnion (Fernandes),[7] Chang liver, clonal HeLa HB_2, McCoy synovial, Henle's intestine, bovine kidney, Chang conjunctiva, NCTC A2414 clonal human skin, human lung developed in the presence of tobacco smoke condensate (HL-S, Nakanishi[10]), and kitten lung (KL, Nakanishi). Trypsin was not used to subculture these cell lines. A rubber policeman was employed to scrape the cells from the glass. These were dispersed with a 30-ml. syringe fitted with a 15-gauge needle, and a final suspension of 10^5 cells per milliliter was prepared. Effective dispersion of single cells was obtained by this method.

Nutrient medium consisted of Eagle's medium containing 100 units of penicillin and 0.1 mg. of neomycin per milliliter with 10 per cent horse serum. The horse serum was inactivated for 30

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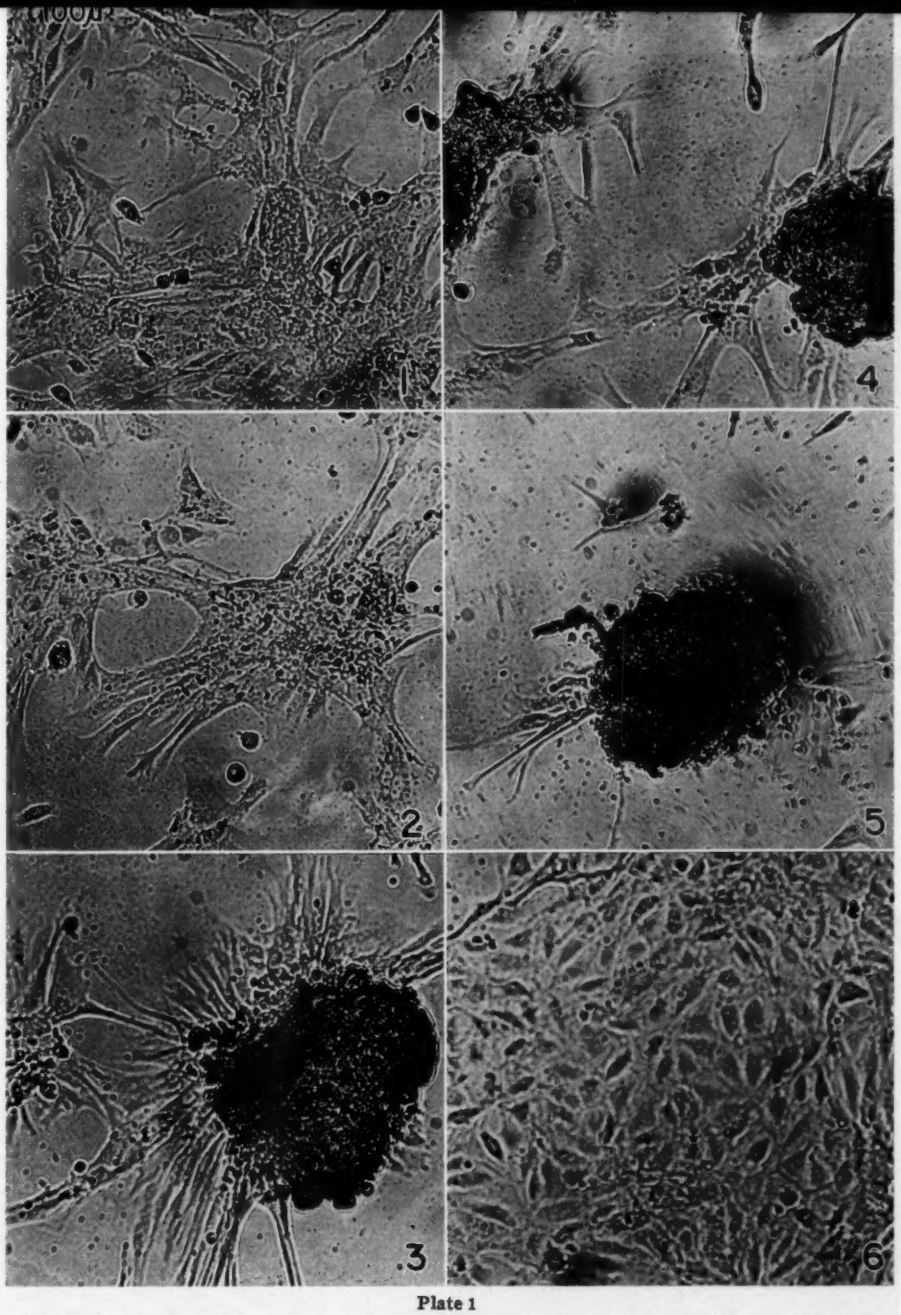

Plate 1

Trypsinized lamb kidney cells. Ordinary light microscopy, T60 flasks; scale of magnification, 100 μ

Fig. 1 and 2—Increased granularity and shrinking of cells 24 and 48 hours, respectively, after inoculation of approximately 100 t.c.i.d.$_{50}$ of BTV.

Fig. 3 and 4—Holes and the formation of aggregate masses of cells 72 and 96 hours after inoculation of the virus.

Fig. 5—An aggregated mass and cellular debris 120 hours following inoculation of the virus.
Fig. 6—Culture control photographed at the same time as figure 5.

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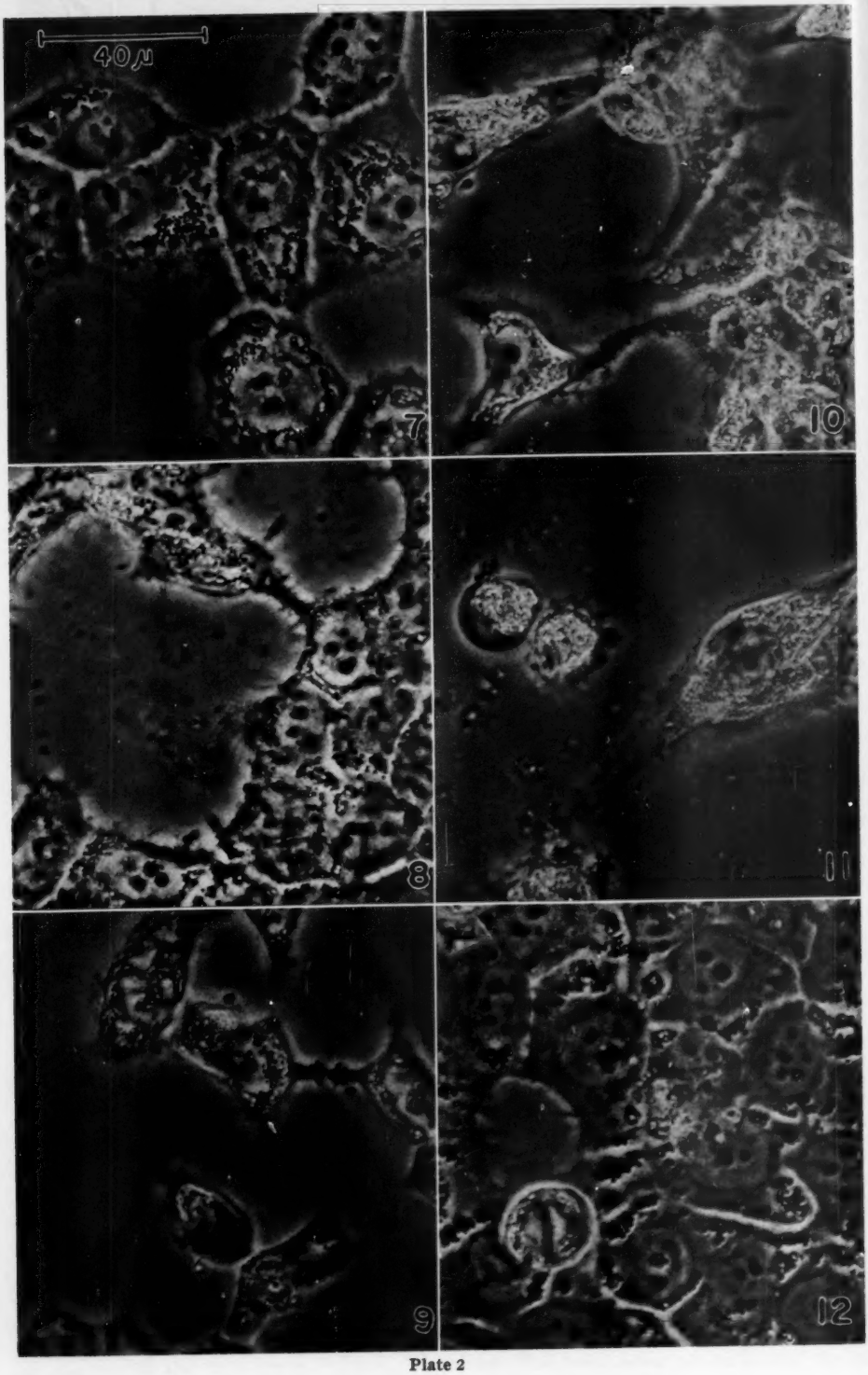

Plate 2

minutes at 56 C. A trypsin solution (Difco 1:250) was used, 0.25 per cent in Gey's BSS without calcium and magnesium, sterilized by Seitz filtration, and kept frozen at −20 C. The pH was adjusted to 7.8 by the addition of N/10 sodium hydroxide after sterilization. For staining and phase contrast microscopy, Leighton tubes and Rose chambers [14] were employed.

Neutralization Tests.—Serum neutralization tests in tissue cultures were done by inoculating tubes with 0.1 ml. of serum-virus mixture. A constant amount of virus was mixed with serial twofold dilutions of immune serum and the mixture remained at room temperature for one hour. The serum-neutralization effect was recorded when approximately 100 50-per cent tissue culture-infected doses (100 t.c.i.d.$_{50}$) of virus was seen to produce a 4$^+$ degeneration in the virus control that was carried out simultaneously.

RESULTS

After treatment with penicillin and neomycin, T60 flasks containing lamb kidney cells were inoculated with 1 ml. of blood obtained from a sheep in the acute phase of the disease. Small foci of degeneration, characterized by shrinking of cells and increased granularity, were first observed after 24 to 36 hours following inoculation. As the injury increased, holes appeared in the cell sheet and most of the cells became detached from the glass. Some seemed to form aggregate masses of dead cells as shown on plate 1. This occurred at the end of the fifth or sixth day. After two passages in trypsinized lamb kidney cells, the virus was subsequently passed into human amnion cells (Fernandes strain). The cytopathogenic effect of the BTV in this cell line differed from the one produced in the lamb kidney cells. Using both stained and unstained cover-slip preparations, a study of the cytopathogenic effect of BTV in this cell line was made at specific time intervals. Following inoculation with approximately 100 t.c.i.d.$_{50}$ of virus, cytopathogenesis was first recognized in living cultures on the first day by the shrinking and the rounding-up of cells, increased granularity, and the production of optically dense nuclei. Holes began to appear in the epithelial sheet on the second day, and some cells assumed a fibroblast-like shape. Finally, the cells separated from one another, became detached from the glass, and floated in the medium. These cellular changes, photographed on Rose chamber cover slips with phase contrast microscopy, are illustrated (plate 2).

At similar stages of infection, upon staining by Jacobson's method,[11] the cells were found to possess distorted and pyknotic nuclei and a basophilic cytoplasm. They presented irregular shapes, some showing a fibroblast-like type. These aspects of the preparations are shown (plate 3). Thus far, no syncytial structures, giant cells, or inclusions could be demonstrated at any stage of virus multiplication *in vitro*.

These studies have also included the preparation and analysis of phase contrast cine records which will form the subject of another paper.

Propagation of the Virus.—The viruses isolated from lamb kidney tissue cultures and the egg-adapted strain were passed serially in human amnion cells (Fernandes strain). Titrations of these viruses were made by inoculation of 0.1 ml. of decimal dilutions into each of five tubes of lamb kidney and human amnion cells. The 50 per cent cytopathogenic end point (t.c.i.d.$_{50}$) was calculated by the method of Reed and Muench.[12] Replicate titrations of virus-containing fluids of various tissue culture passages showed t.c.i.d.$_{50}$ of $10^{-4.2}$ per 0.1 ml. At the time of writing, eight

Legend for Plate 2 on Opposite Page

Human amnion (Fernandes strain). Dark phase microscopy, Rose chambers; scale of magnification, 40 μ.

Fig. 7—Rounding up and granularity of cells 24 hours after inoculation of approximately 1,000 t.c.i.d.$_{50}$ of BTV.

Fig. 8—Holes and shrinking of cells (some being the fibroblast-like type) 48 hours after inoculation.

Fig. 9, 10, and 11—Progressive cellular destruction, with increasing granularity and shrinking of cells 72, 96, and 120 hours, respectively, following inoculation of the virus. Optically dense nuclei are shown in figures 9 and 10.

Fig. 12—Culture control photographed at the same time as figure 11.

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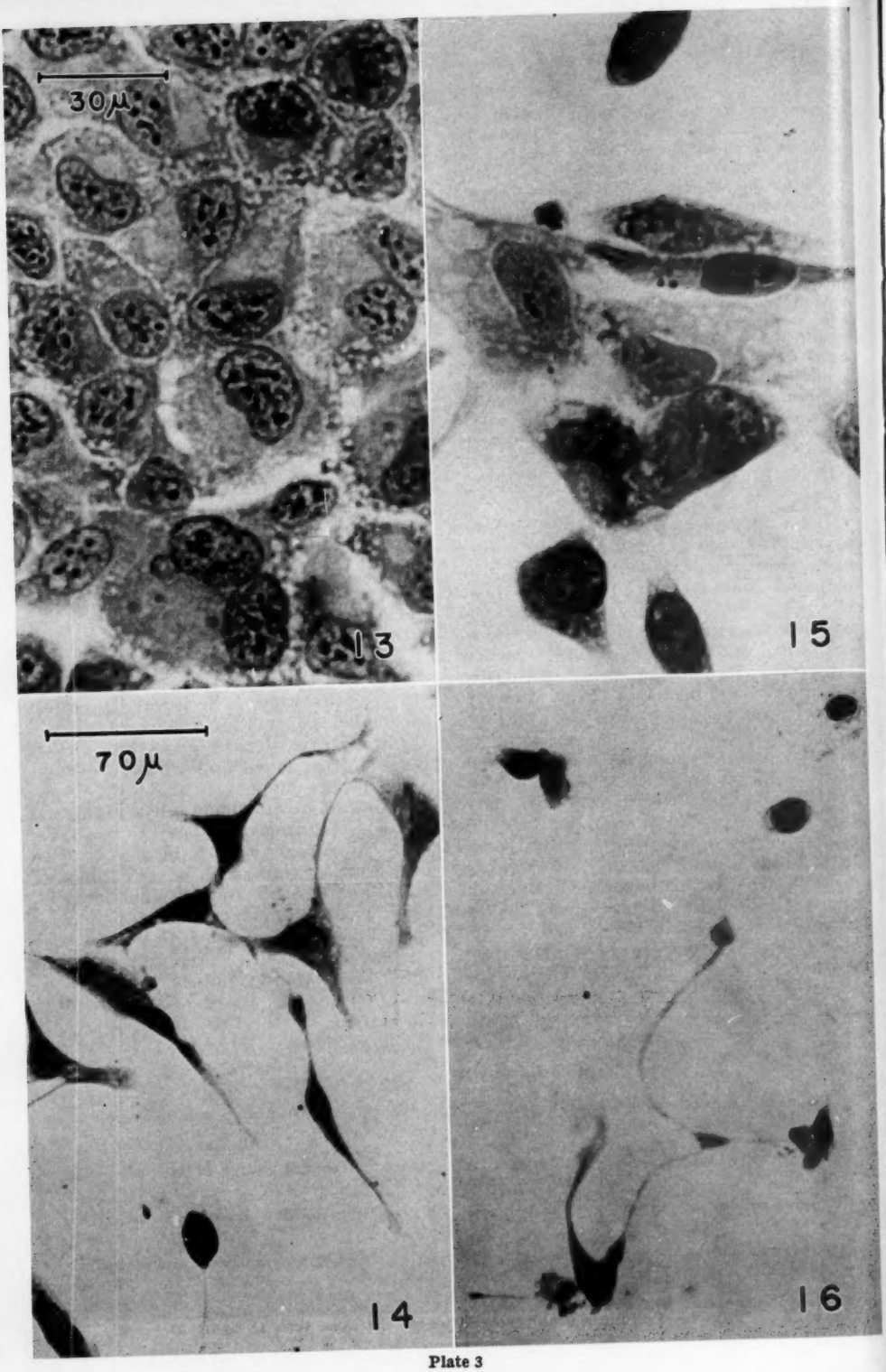
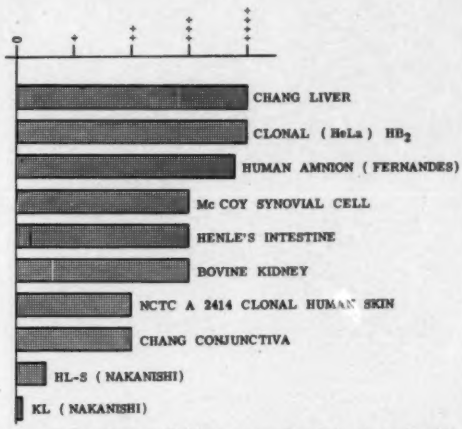


Plate 3

passages of the egg-adapted virus in tissue culture had been made, each inoculum consisting of the preceding culture harvested at the time when maximum degeneration occurred. By this transfer, when the original inoculum had undergone a theoretical dilution of 10^{-16}, the virus-containing fluid still possessed a t.c.i.d.$_{50}$ of approximately 10^{-4}. Therefore, there is ample evidence of viral multiplication.

Susceptibility of Various Strains to Bluetongue Virus.—The cell lines studied showed different susceptibilities to BTV (graph 1). The Chang liver strain, clonal HeLa HB_2, and human amnion (Fernandes) are the most susceptible strains compared with the HL-S (Nakanishi) and KL (Nakanishi) strains, which are not affected morphologically by BTV. Plates 4 and 5 show the comparative values of the susceptibility of those strains of cells to BTV infection as reflected by cytopathogenic changes recorded on the fourth day. Plate 6 points out the serial aspects presented after inoculation of the virus in the McCoy synovial cells and bovine kidney strain, in which greater increase of virus titer was found. A detailed report on these observations and on the behavior of the virus-cell systems in all previously mentioned strains is in preparation.

Effect of Bluetongue Virus Adapted to Tissue Culture on Embryonating Eggs.—The virus-content fluid of each passage of the egg-adapted strain of BTV was inoculated in 8-day embryonating eggs according to the technique prescribed by Alexander.[1] The eggs were inoculated in the yolk sac with 0.3 ml. of 10^{-2} dilution of the virus. The eggs were then incubated for 24 hours at 35 C. After the first day, the incubation temperature was changed to 32 C. The infected embryos died on the third or fourth day after inoculation. When dead or dying, they had the characteristic cherry red color described by Mason *et al.*[9] No changes were observed in the infectivity of the virus against chicken embryos. The virus of the eighth passage in tissue culture still was able to kill the embryos during the third or fourth day following inoculation.



Graph 1—Susceptibility of various strains of cells to BTV infection as reflected by cytopathogenic changes recorded on the fourth day. Total injury is represented by 4+.

Neutralization Tests.—Using a virus suspension diluted to contain approximately 100 t.c.i.d.$_{50}$, a neutralization end point of 1:4 was found for the immune serum with which these studies were done. The undiluted normal serum did not affect the cells, and the virus alone produced a 4+ degeneration on the fifth day following inoculation. Neutralization tests were conducted for the virus isolated on lamb kidney tissue cultures and for the third passage of this virus in the same cell system. These tests were also carried on for the virus of the fifth and eighth passages on human amnion cells. The results of one test are shown (table 1).

Legend for Plate 3 on Opposite Page

Scale of magnification for figures 13 and 15 is shown on the upper left area of figure 13, and for figures 14 and 16, on the upper left area of figure 14; Jacobson's methods.

Fig. 14 and 15—Human amnion cells (Fernandes strain) 72 hours after inoculation of approximately 1,000 t.c.i.d.$_{50}$ of BTV. Notice the presence of distorted and pyknotic nuclei, basophilic cytoplasm, and the irregular shape of the cells, some being the fibroblast-like type.

Fig. 16—Final phase of cellular destruction. 120 hours after viral infection.

Fig. 13—Culture control photographed at the same time as figure 16.

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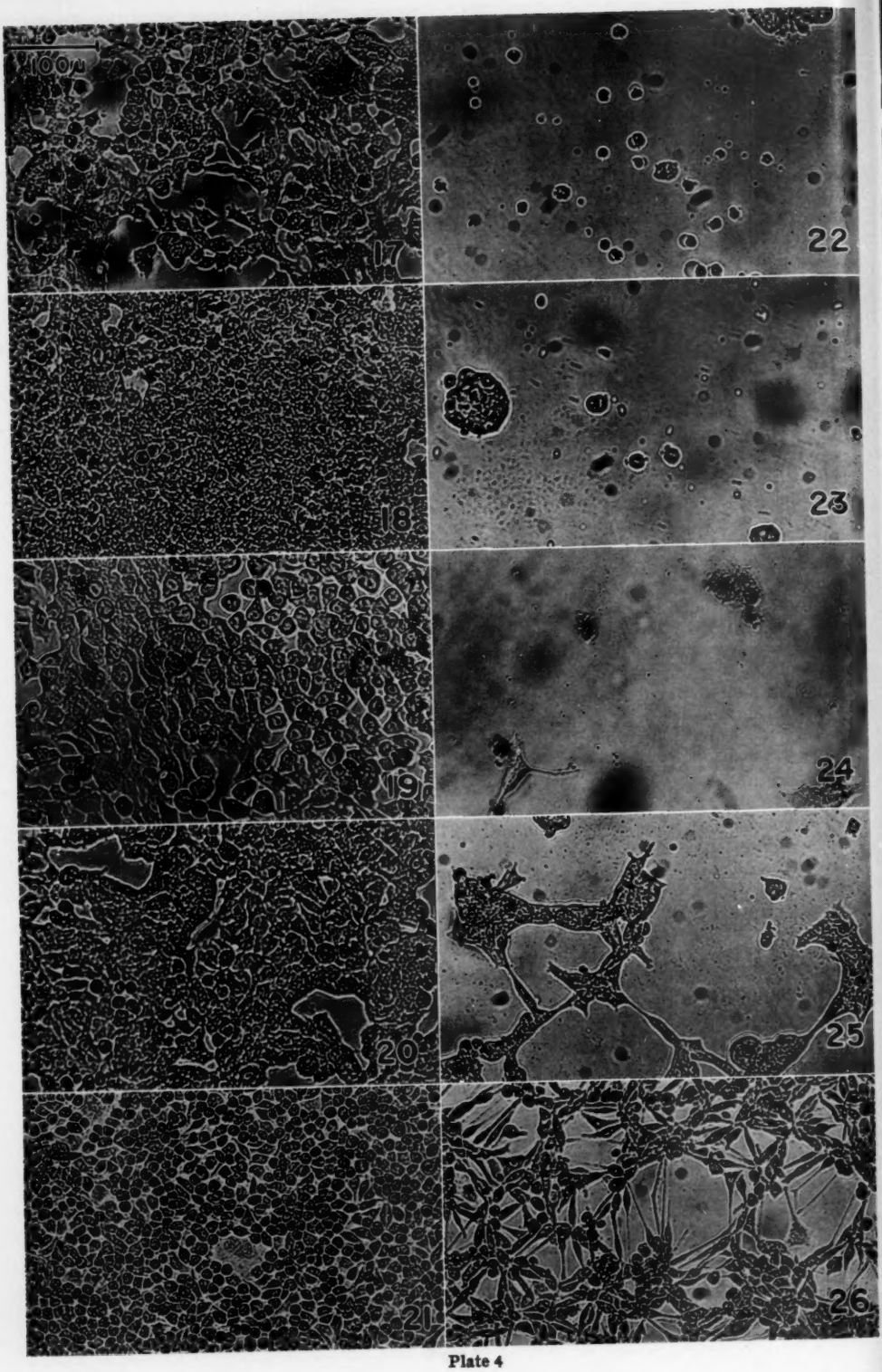

Plate 4

TABLE 1—Serum-Virus Neutralization in Vitro

Serum dilution	Virus	Days after inoculation					
		1	2	3	4	5	6
Undiluted	100 t.c.i.d.$_{50}$	0	0	0	0	0	0
1:2	100 t.c.i.d.$_{50}$	0	0	0	0	0	0
1:4	100 t.c.i.d.$_{50}$	0	0	0	0	0	0
1:8	100 t.c.i.d.$_{50}$	0	∓	+	++	+++	++++
1:16	100 t.c.i.d.$_{50}$	∓	+	++	+++	+++	++++
1:32	100 t.c.i.d.$_{50}$	∓	+	++	+++	+++	++++
Undiluted normal serum	100 t.c.i.d.$_{50}$	∓	+	++	+++	++++	++++
No serum	100 t.c.i.d.$_{50}$	∓	+	++	+++	++++	++++
Undiluted normal serum	No virus	0	0	0	0	0	0

DISCUSSION

It has been shown in the data presented that BTV can be isolated and propagated in tissue culture with characteristic cytopathogenic effect. According to the review by Enders,[5] three types of viral activity are suggested. Changes due to the virus are grouped as follows: (1) those causing only degeneration, manifested by rounding-up of cells, or presenting irregular outlines, increasingly granular cytoplasm, swelling of cells, pyknosis, fragmentation of nuclear chromatin, cell death, and disintegration; (2) those inducing formation of inclusion bodies and cell degeneration; (3) those inducing formation of multinucleated cells or syncytial masses and degeneration, exhibiting a tendency to produce foci of infected cells with or without inclusion body formation. In compliance with these designations, BTV is placed in the first group.

The relatively rapid growth of BTV in tissue culture made identification of the virus possible in as little as two or three days after inoculation. Following experimental infection of sheep, the incubation period generally varied from two to four days, but in some cases was as long as 18 days.[3] White mice, after intracerebral inoculation of the virus, died on the third, fourth, or fifth day, depending on the strain of BTV.[17] In the first passage of the virus, developing chicken embryos usually died on the sixth day. These facts, in addition to some undetermined factors, such as immunity of individual sheep and their variable reaction to the virus, proved that tissue culture is a creditable procedure in the diagnosis and study of bluetongue of sheep.

Another advantage of the use of tissue culture techniques is the fact that tests can be carried out in a small laboratory without the problems of the isolation of inoculated animals (sheep and laboratory animals), the need for incubators held at 35 and 32 C., and all the other technical complications essential for the maintenance of a "classical" microbiological laboratory.

It is important, however, to determine if the virus can be easily recovered and identified from tissues of infected sheep, since the virus is present in the blood in readily detectable amounts only during the acute febrile stage of the disease, and further investigations should be made to determine if the virus can be practicably and repeatedly isolated from the blood of infected sheep.

The *in vitro* neutralization test, with its type specificity and easy performance, can be used for bluetongue diagnostic purposes in tissue culture.

The preparation of a vaccine from the tissue culture-adapted virus seems to merit

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Ordinary light microscopy, T30 flasks; scale of magnification, 100 μ.

Fig. 22 to 26—The injury in various cell lines is apparent on the fourth day after inoculation of approximately 100 t.c.i.d.$_{50}$ of BTV.

Fig. 17 to 21—Culture controls; Chang liver (17 and 22); clonal HeLa HB$_2$ (18 and 23); human amnion, Fernandes strain (19 and 24); Henle's intestine (20 and 25); McCoy synovial cells (21 and 26).

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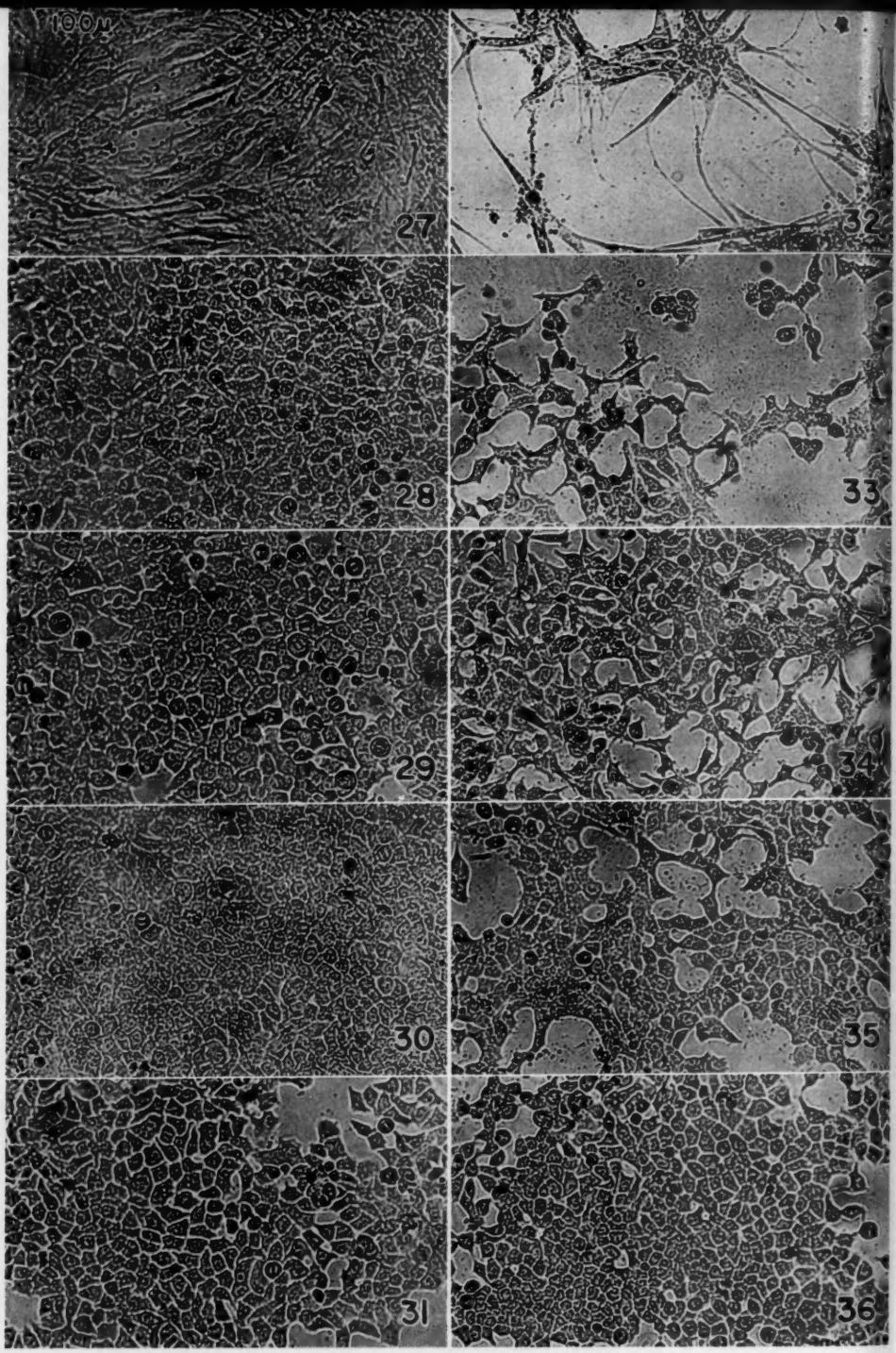

Ordinary light microscopy, T30 flasks; scale of magnification, 100 μ.

Fig. 27 to 31—Culture controls. Fig. 32 to 36—The injury presented in various cell lines on the fourth day after inoculation of approximately 100 t.c.i.d.$_{50}$ of BTV; bovine kidney (27 and 32); Chang conjunctiva (28 and 33); NCTC A 2414 clonal human skin (29 and 34); HL-S, Nakanishi (30 and 35); KL, Nakanishi (31 and 36).

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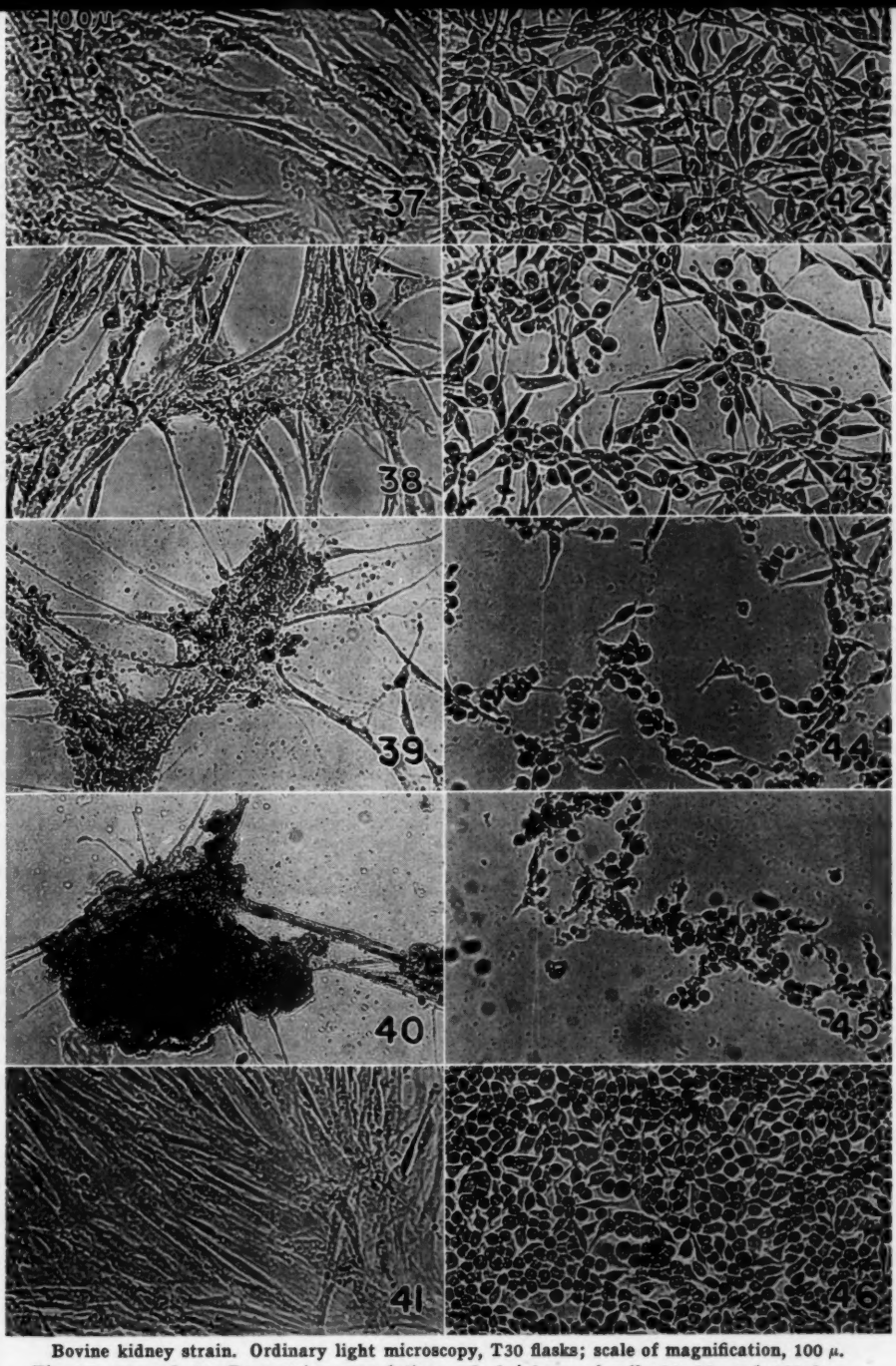

Bovine kidney strain. Ordinary light microscopy, T30 flasks; scale of magnification, 100 μ.

Fig. 37, 38, and 39—Progressive granulation and shrinkage of cells 24, 72, and 120 hours, respectively, after inoculation of approximately 100 t.c.i.d.$_{50}$ of BTV.

Fig. 40—Aggregated masses of cells 144 hours after inoculation of the virus.

Fig. 41—Culture control photographed at the same time as figure 40.

Fig. 42 to 46—McCoy synovial cells.

Fig. 42 to 45—Progressive granulation, rounding up, shrinking, and death of cells 48, 72, 120 and 144 hours, respectively, after inoculation of approximately 100 t.c.i.d.$_{50}$ of BTV.

Fig. 46—Culture control photographed at the same time as figure 45.

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investigation because of the advantages listed, even though the egg-prepared vaccine [2] has proved to be efficient for the immunization of sheep.

SUMMARY AND CONCLUSIONS

Bluetongue virus obtained from the blood of a sheep in the acute phase of the disease was isolated on monolayers of trypsinized lamb kidney cells and was propagated both in these cells and in a strain of human amnion (Fernandes), showing cytopathology with increasing virus titer.

An egg-adapted virus was passed serially eight times in tissue culture, assuming at this point a theoretical titer dilution of 10^{-16}, which offered ample evidence of viral multiplication.

The susceptibility of ten cell lines has been studied. Cells were observed with phase contrast microscopy and after staining in order to determine the course of the lesions caused by the virus.

Time-lapse, phase-contrast cinematography also was used in the study of the cell-virus system. Thus far, no specific lesion has been found.

Cytological degeneration in cell cultures can be neutralized by immune serum and, therefore, is useful in the diagnosis of bluetongue.

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A Comparison of the Anthelmintic Efficiency of Purified and N. F. Phenothiazine

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IN ATTEMPTING to develop a more efficacious formulation and account for anthelmintic failures of phenothiazine, the authors have completed intensive trials on the influence of particle size on anthelmintic efficiency of phenothiazine.[1,2] From this work it was concluded that where species of Ostertagia, Trichostrongylus, and Nematodirus are the nematodes of greatest importance, a phenothiazine preparation having a mean particle diameter of 2 μ or less (specific surface of 25,000 cm.^{2}/Gm. or more) was most desirable.[2] The preparation and formulation of such a product would increase milling costs and the increased bulk might lead to difficulties in administration.

During the 1956 trials,[2] a preparation of purified phenothiazine having a mean particle diameter of 4.8 μ (10,000 cm.^{2}/Gm.) was found to have an anthelmintic efficiency equal to that of N.F. phenothiazine of 2.0 μ or less and significantly greater efficiency than N.F. phenothiazine of a comparable mean particle diameter. Since preparations of this particle size are relatively easy to produce and most of the problems associated with the increased bulk of finer particle size are eliminated, further work with purified phenothiazine was indicated.

The present paper is a report of trials conducted in 1957 using lambs and laboratory mice, which confirms and extends the previous results.[1,2]

MATERIALS AND METHODS

Experimental work was conducted in two phases. In the first, range lambs with natural worm infections were used and, in the second, laboratory mice experimentally infected with *Nematospiroides dubius* Baylis, 1926, were employed as experimental material.

The phenothiazine used in both the sheep and mouse trials was from the same stock and was characterized* as follows: (1) purified phenothiazine, mean particle diameter 14.5 μ, specific surface 3,312 cm.^{2}/Gm. (P 14.5 μ); (2) purified phenothiazine, mean particle diameter 6.6 μ, specific surface 6,940 cm.^{2}/Gm. (P 6.6 μ); (3) commercial N.F. phenothiazine, mean particle diameter 10.0 μ, specific surface 4,412 cm.^{2}/Gm. (N.F. 10.0 μ); and (4) commercial N.F. phenothiazine, mean particle diameter 2.6 μ, specific surface 17,480 cm.^{2}/Gm. (N.F. 2.6 μ).

Fecal egg counts were made by use of the McMaster technique.[3,5] These counts were recorded *in ova* per gram of normal sheep feces and, in the case of mice, *in ova* per gram of dried feces.

In the sheep experiment, 47 range lambs were allotted to five groups in such a manner that the mean egg counts per gram of feces, as well as the range of individual counts, were similar in each group. Six egg counts were made from each lamb over a period of one month prior to treatment, during which time the animals were kept in a drylot and fed alfalfa hay. The lambs were then treated as follows: group 1 (10 lambs), no treatment; group 2 (10 lambs), 25 Gm. P 14.5 μ; group 3 (8 lambs), 25 Gm. P 6.6 μ; group 4 (10 lambs), 25 Gm. N.F. 10.0 μ; and group 5 (9 lambs), 25 Gm. N.F. 2.6 μ.

The drug was prepared by weighing individual doses, mixing with 10 to 25 ml. of a 1 per cent Tween 80 solution as a wetting agent, and adding sufficient water to allow administration by stomach tube.

On the second, fourth, and sixth days following treatment, egg counts were made from all animals. The lambs were necropsied on the sixth day after treatment and total worm counts and species percentile composition of the various groups determined as previously described.[1]

In the mouse trials, two comparable experiments were conducted. One hundred mice were each inoculated with 75 infective larvae of *N. dubius* and randomly placed in ten groups of 10 mice each. Five groups were employed in each of the experi-

From the University of California, School of Veterinary Medicine, Davis (Baker, Allen, and Douglas) and the University of California Field Station, Hopland (Longhurst).

This investigation was conducted as a part of Western Regional Research Project W-35, "Internal Parasites of Ruminants."

The authors express their appreciation to the Dow Chemical Co., Midland, Mich., who supplied the phenothiazine as well as a part of the funds necessary for completion of the project.

* Particle size measurements were made in the Department of Engineering, University of California, Berkeley, by use of the Fisher Sub-Sieve Sizer.

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ments which, except for the time of necropsy, were duplicates of each other and were run concurrently.

Forty days after inoculation, the desired groups were given rations containing the appropriate phenothiazine preparation at a concentration of 0.25 per cent. Fifteen days prior to feeding the mice the medicated rations and continuing for 60 days, at which time maximum effect of the drug on ovogenesis had occurred, fecal samples were collected from all groups three times each week. The animals of one group in each experiment were not fed phenothiazine and served as controls.

All animals in the first experiment were necropsied 51 days after being placed on the medicated ration, and those in the second experiment were necropsied 70 days after treatment started. Total worm counts were made at necropsy by the method of McGee *et al.*[4]

RESULTS

SHEEP TRIAL

Fecal Egg Counts.—The results of the six pretreatment and post-treatment egg counts are given as the average number of eggs per gram of feces per lamb (table 1).

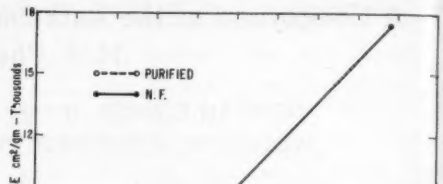
TABLE 1—Average Fecal Egg Counts of Lambs

Group	Control	P 14.5	P 6.6	N.F. 10.0	N.F. 2.6
Pretreatment	609	628	661	612	836
Post-treatment	728	9	4	129	11
Percentage reduction	—20	99	99	79	99

It is apparent that, insofar as ovogenesis is concerned, P 14.5 μ, P 6.6 μ, and N.F. 2.6 μ are comparable, while N.F. 10.0 μ is less efficacious.

Number of Worms in Lambs at Necropsy.—The average number of worms and percentage of reduction found at necropsy in the abomasums and small intestines of the lambs in each group are shown (table 2; graphs 1, 2).

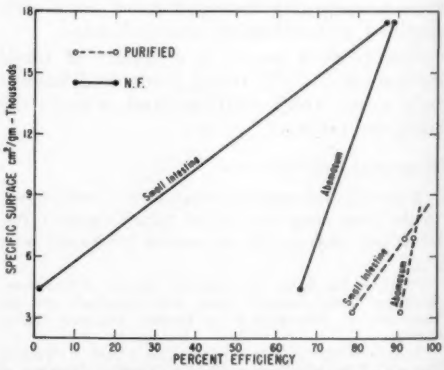
Analysis of the data from abomasums indicated that when the groups treated with P 14.5 μ, P 6.6 μ, and N.F. 2.6 μ were compared to the control group, the differences were significant to the 1 per cent level. The group treated with N.F. 10.0 μ, when compared to the control group, differed significantly at the 5 per cent level. No other differences were found to be significant. However, the differences between the following groups would have been expected to occur by chance only 5 to 10 per cent of the time: P 14.5 μ and N.F. 10.0 μ; P 6.6 μ and N.F. 10.0 μ; and N.F. 2.6 μ and N.F. 10.0 μ. The difference between groups



Graph 1—The relationship between particle size and anthelmintic efficiency of purified and N.F. phenothiazine in the small intestines and abomasums of lambs.

treated with P 14.5 μ and P 6.6 μ would have been expected to occur by chance 30 to 40 per cent of the time.

Analysis of data from the small intestines indicated that the groups which received P 14.5 μ, P 6.6 μ, and N.F. 2.6 μ differed from the control group at the 1 per cent level, while the group given N.F. 10.0 μ did not differ from the controls at any level of significance. The data from groups receiving P 14.5 μ and P 6.6 μ differed significantly at the 5 per cent level, while the differences between groups fed P 6.6 μ and N.F. 2.6 μ would be expected to occur by chance 30 to 40 per cent of the time.



Graph 2—The relationship between particle size and anthelmintic efficiency of purified and N.F. phenothiazine measured in terms of total worm counts in the abomasums and small intestines of lambs.

TABLE 2—Average Number of Worms in Lambs and Percentage Reduction at Necropsy

Treatment	Abomasum	Reduction (%)	Small intestine	Reduction (%)	Total	Reduction (%)
Control	813	0	1,320	0	2,133	0
P 14.5 μ	72	91	271	79	343	84
P 6.6 μ	51	94	110	92	161	92
N.F. 10.0 μ	280	66	1,302	1	1,582	26
N.F. 2.6 μ	88	89	178	87	266	88

Analysis of data from total worm counts indicated that the differences in anthelmintic efficacy of P 14.5 μ and N.F. 2.6 μ; P 6.6 μ and N.F. 2.6 μ; and N.F. 10.0μ and no treatment were not significant. All other differences were significant to the 1 or 5 per cent level.

Species of Worms Recovered at Necropsy.—The data pertaining to species composition of worm populations in the five groups of lambs are shown (table 3). *Strongyloides papillosis* was not included in the data presented in tables 1 and 2, because phenothiazine is known to have little or no anthelmintic action against this nematode.

MOUSE TRIALS

Fecal Egg Counts.—In order to compare the data from different groups, linear regression lines were calculated for groups given P 14.5 μ, P 6.6 μ, and N.F. 2.6 μ. From these calculations, the number of days of treatment required for the egg counts to reach zero (table 4) was computed. This type of calculation could not be applied to groups treated with N.F. 10.0 μ since, even though egg counts decreased to 40 or 50 per cent of pretreatment levels, a plateau was reached after the third week and no further reduction occurred. Control groups maintained nearly constant levels of egg production.

TABLE 3—Species Distribution by Organ—Figures are Percentage of Total Worms Present

Species	Group 1	2	3	4	5
Abomasums					
Ostertagia circumcincta ♂	29	52	28	23	33
Ostertagia trifurcata ♂	1	4	0	1	3
Ostertagia spp. ♀	39	31	58	34	43
Trichostrongylus axei ♂	8	2	0	14	4
Trichostrongylus vitrinus ♂	4	1	14	6	2
Trichostrongylus spp. ♀	19	10	0	22	15
Small intestines					
Trichostrongylus vitrinus ♂	24	20	26	33	29
Trichostrongylus colubriformis ♂	2	3	4	2	2
Trichostrongylus spp. ♀	28	20	37	40	22
Nematodirus spathiger	26	1	0	10	2
Nematodirus filicolis	12	23	12	5	19
Immature Nematodirus spp.	4	13	14	6	10
Strongyloides papillosis	4	20	7	4	16

During the course of the experiment, the mice receiving the purified phenothiazine preparations consumed only about 75 per cent as much feed as did those given N.F. phenothiazine. This difference is assumed to be a problem of palatability. In any case, in those groups fed P 14.5 μ and P 6.6 μ, only about 75 per cent as much phenothiazine was consumed, and this would tend to make the difference between results obtained with purified and N.F. materials even more striking.

TABLE 4—Days Required for Egg Counts of Treated Mice to Reach Zero

Treatment	Experiment 1	Experiment 2
Control	∞*	∞
P 14.5μ	19.9	17.7
P 6.6 μ	16.0	15.0
N.F. 10.0 μ	∞	∞
N.F. 2.6 μ	16.7	17.6

* Egg counts did not reach zero and were maintained at a constant value.

Total Worm Counts.—Data pertaining to the average worm count per mouse in each group are presented (table 5).

Data from experiment 1 (table 5) appear to indicate that P 14.5 μ and P 6.6 μ material have a comparable efficiency to N.F. 10.0 μ and N.F. 2.6 μ material, respectively. This same effect was observed in a previous report [1] when N.F. phenothiazine of different particle size was used in similar experiments. In that case, it was demonstrated that mice given different particle size material had similar worm counts at seven days of treatment, although the dif-

TABLE 5—Average Worm Count Per Mouse at Necropsy

Treatment	Experiment 1* Worms (No.)	Efficiency (%)	Experiment 2† Worms (No.)	Efficiency (%)
Control	50.3	0.0	52.5	0.0
P 14.5 μ	27.0	46.3	0.8	98.5
P 6.6 μ	9.1	81.9	3.3	93.7
N.F. 10.0 μ	27.4	45.5	23.0	55.9
N.F. 2.6 μ	11.7	76.7	4.5	91.4

* Necropsied after 51 days of treatment. † Necropsied after 70 days of treatment.

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ference in effect was quite noticeable in terms of ovogenesis and after prolonged treatment it also became apparent in total worm count.

Examination of experiment 2 (table 5) reveals that after a longer period of treatment P 14.5 μ, P 6.6 μ, and N.F. 2.6 μ all seem to be of equal anthelmintic efficiency and are much more efficacious than N.F. 10.0 μ.

DISCUSSION

The present experiments confirm the previous results [1,2] concerning the inverse relationship between particle size and anthelmintic efficiency of phenothiazine. In addition, the data on N.F. phenothiazine (table 2; graphs 1, 2) indicate the importance of particle size as a possible cause of anthelmintic failure in field cases. In a previous report,[2] it was shown that there exists a linear relationship between specific surface (cm.^{2}/Gm.) of N.F. phenothiazine and anthelmintic efficiency between 5,000 cm.^{2}/Gm. and 25,000 cm.^{2}/Gm., corresponding to mean particle diameters of about 10.0 μ to less than 2.0μ. It was also shown at that time, as well as in the present experiments, that N.F. phenothiazine was more efficient in the abomasum than in the small intestine and that this difference increased with an increase in particle size. Above a mean particle diameter of 10.0 μ (specific surface 5,000 cm.^{2}/Gm. or less), the efficiency of N.F. phenothiazine has been found to drop rapidly.[2] It is therefore the contention of the authors that this accounts for the ineffectiveness of the N.F. 10.0 μ material in the small intestines of lambs under the conditions of the present trial and perhaps in many clinical outbreaks.

The evidence for superiority of purified phenothiazine used in this trial over N.F. phenothiazine would appear to be well substantiated. The data from sheep closely resemble those in the previous report [2] with regard to both purified and N.F. phenothiazine, while data from mice are directly comparable. Comparison of previous data [2] and the present sheep data indicates that if purified phenothiazine is used, a suitable product for sheep could be prepared with a specific surface as low as 6,000-7,000 cm.^{2}/Gm. (mean particle diameter of 6 μ or less) as compared with N.F. phenothiazine which should have a specific surface of at least 25,000 cm.^{2}/Gm. (mean particle diameter of 2 μ or less).

The authors are unable to account for the difference between the two phenothiazine preparations—N.F. phenothiazine is reported to contain approximately 96 per cent phenothiazine; the remainder is made up of gums, catalysts such as iodine, and many undetermined substances. The material may be purified by several methods, and even these are not entirely free of contaminating substances. This in itself presents a problem which must be studied, for if the increased activity is due either to the removal of one or more of the contaminating materials, or to the addition of others during purification, then it is possible, if not probable, that the results reported in this paper are applicable only to phenothiazine purified by the method used in preparation of the experimental material.* An additional possibility is that methods used for phenothiazine analysis are inadequate and, as a result, materials present in N.F. preparations which do not possess anthelmintic action are determined as phenothiazine.

SUMMARY

The anthelmintic efficiency of purified phenothiazine preparations having a mean particle diameter of 14.5 μ and 6.6 μ were compared with preparations of N.F. phenothiazine having mean particle diameters of 10.0 μ and 2.6 μ. Trials were conducted in sheep with naturally acquired worm infections and in laboratory mice experimentally infected with *Nematospiroides dubius*.

The purified material of 6.6 μ mean particle diameter (specific surface 6,940 cm.^{2}/Gm.) gave comparable results to an N.F. phenothiazine preparation having a mean particle diameter of 2.6 μ (specific surface 17,480 cm.^{2}/Gm.). The purified material having a mean particle diameter of 14.4 μ (specific surface 3,312 cm.^{2}/Gm.) was only slightly less efficient than that having a mean particle diameter of 6.6 μ and the N.F. preparation having a mean particle diameter of 2.6 μ, but was markedly more efficient than the N.F. phenothiazine having a mean particle diameter of 10.0 μ (specific surface 4,412 cm.^{2}/Gm.).

All preparations were more efficient

* The method was developed and has been used in commercial production by the Dow Chemical Company, Midland, Mich.

against species of worms occurring in the abomasums than against those in the small intestines of sheep.

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[1] Douglas, J. R., Baker, N. F., and Longhurst, W. M.: The Relationship Between Particle Size and Anthelmintic Efficiency of Phenothiazine. Am. J. Vet. Res., *17*, (April, 1956): 318–323.

[2] Douglas, J. R., Baker, N. F., and Longhurst, W. M.: Further Studies on the Relationship Between Particle Size and Anthelmintic Efficiency of Phenothiazine. Am. J. Vet. Res., *19*, (Jan., 1959): 201–205.

[3] Gordon, H. McL., and Whitlock, H. V.: A New Technique for Counting Nematode Eggs in Sheep Feces. J. Council Scient. Indust. Res., Australia, *12*, (1939): 50–52.

[4] McGee, G. M., Russomanno, R., and Sandground, J. H.: An Expeditious Method for Counting Trichostrongylids and Other Helminths of the Small Intestine. J. Parasitol., *43*, (1957): 315–317.

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Scientific Notes

Foundation Announces Establishment of Research Advisory Service on Information Processing

The National Science Foundation announced Nov. 17, 1958, the establishment of a Research Information Center and Advisory Service on information processing to be operated jointly by the Foundation and the National Bureau of Standards.

The new service is designed to bring together research and development data on methods and equipment for the automatic processing of scientific information. In addition, the service is designed to foster closer cooperation among groups in industry, private foundations, universities, professional societies, and the federal government that are concerned with developing and improving methods for rapid and efficient handling of large volumes of information.

Initiation of this service is a part of a broad program being developed by the National Science Foundation to improve the quality of scientific information services and to shorten the time spent by the scientists in searching through literature. Other phases of the extensive program include the support of scientific publications, translations of foreign scientific literature, and the support of information and data-processing centers.

Organizations and individuals engaged in research and development activities or planning programs in these areas may request further information about the new service and the procedures to be followed in requesting advice or information by writing to, the Research Information Center and Advisory Service on Information Processing, Program for Documentation Research, National Science Foundation, Washington 25, D.C.

Report Made on American Scientists' Knowledge of Foreign Languages

Three out of every four American scientists have indicated knowledge of at least one foreign language according to a study released in November by the National Science Foundation.

German and French, in that order, were reported known most frequently, reflecting the educational requirements for scientific training, particularly at the graduate level. Due to birth or family background, about two percent of the total have some knowledge of Russian; one per cent of Chinese or Japanese. Thus, nearly all American scientists must depend on translations for knowledge of scientific developments reported on in these tongues.

This study was based on information supplied by the 127,000 scientists listed in the National Register of Scientific and Technical Personnel in 1954–1955. The Register is maintained jointly by the Foundation and a number of cooperating professional scientific societies. It is estimated that the Register included more than half of all American scientists, at the time the survey was being made.

Copies of the survey, "Foreign Language Knowledge of American Scientists, 1954–55" are available in limited number from the Public Information Office, National Science Foundation, Washington 25, D.C.

Meetings • • •

• • • International Veterinary Congress. Sixteenth session. Madrid, Spain, May 21–27, 1959. Prof. Pedro Carda A., general secretary, Calle Villanueva 11, Madrid.

U. S. COMMITTEE: Dr. W. A. Hagan, chairman, New York State Veterinary College, Ithaca, N. Y.; Dr. J. G. Hardenbergh, secretary, 600 S. Michigan Ave., Chicago 5, Ill.

• • • Third World Congress on Fertility and Sterility, Amsterdam, Holland, June 7–13, 1959. Dr. L. I. Swaab, Sint Agnietenstraat 4, Amsterdam, Holland, honorary secretary.

• • • Third Pan American Congress of Veterinary Medicine and Ninety-Sixth Annual Meeting, American Veterinary Medical Association, Joint meeting. Kansas City, Mo., Aug. 23–27, 1959. H. E. Kingman, Jr., executive-secretary, AVMA, 600 S. Michigan Ave., Chicago 5, Ill. B. D. Blood, secretary-general. Directing Council, Pan American Congress of Veterinary Medicine, P. O. Box 99, Azul, F.C.N.G.R., Argentina, S.A.

Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.

Escape-Proof Container

An inexpensive, escape-proof container for shipping rats to medical research laboratories has been developed by the Charles River Breeding Labora-

tories. The animals require no attention while in transit and no preparations, such as sedatives or tranquilizers, are needed in preparation for travel.

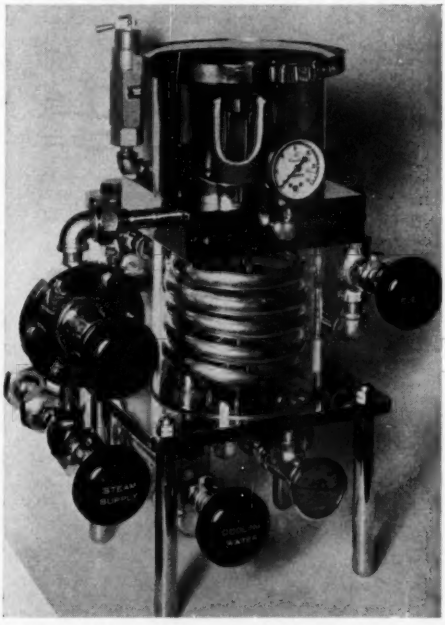
The container is made of corrugated board and is assembled by stapling. Inside, there is wire mesh screening across the bottom and over the ventilation openings located in the front and back of the container. A waxy coating on the sides and top repel moisture, preventing soft wet spots that the animals could chew through. Because of the shape, the containers can be stacked without blocking ventilation.

Dry pellets are supplied as food, and water is provided in sliced potatoes. The animals are on the same bedding they had in the breeding laboratory. The bedding,* deodorizing and self-sanitizing, prevents the rats from becoming a nuisance in planes and at loading areas.

* Sterolit, manufactured by Minerals & Chemicals Corporation of America, is composed of an inert, naturally sorptive mineral that has been chemically processed to make it self-sanitizing and deodorizing.

New Distillation Unit

A new patented distillation unit, Steril-Aqua, is capable of producing sterile, pyrogen-free water from ordinary boiler steam.

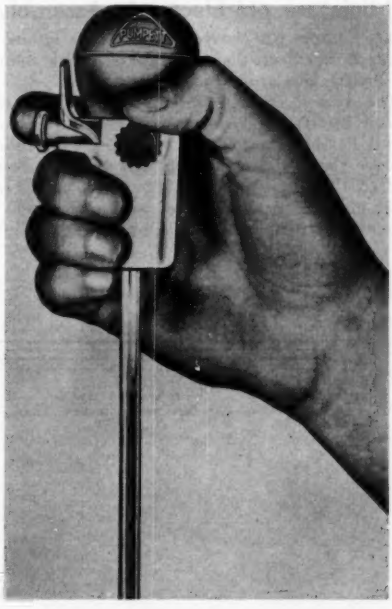


The unit, produced by the Wilmot Castle Company, was developed to provide an economical method of "on-site" production of high purity injectable water. Tests established that water, purposely contaminated to 6,500 p.p.m., can be purified to 0.5 p.p.m. in a single pass. An automatic quality control insures that if water purity falls below a preselected standard, the water will be by-passed by a solenoid valve and returned to waste.

The system operates by using centrifugal force and a hydrophobic separator-filter element to liquid from vapor phase of boiler steam. The complete process includes steam reception, centrifugal separation, vapor-liquid separation, condensation, degassing, and discharge.

Automatic Pipette

A new pipetting device (Pumpett Automatic Pipette Control, produced by Arthur S. LaPine and Co.) eliminates filling the laboratory pipette by mouth suction.



The unit is operated with one hand. The rubber bulb, controlled by the thumb, provides suction and the coarse air control valve is operated by the forefinger. A small internal rubber bulb, depressed by a control knob screw, can be operated independently for microscopic quantities. This bulb accurately controls filling and discharge; the device can replace expensive burettes. The rubber-lined chuck jaws, spring loaded and operated by a lever to clamp or release a pipette, can accomodate all sizes of pipettes.

The device is especially useful for safety where corrosive, toxic, or infectious liquids and sterile or radioactive solutions are handled. It can be completely dismantled for cleaning and sterilizing, and quickly reassembled.

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Radioactive Standards and Sources Set

A new standards and sources set has been made available from the Nuclear-Chicago Corporation.

The set contains a selected group of four certificated radioactive standards in solution and a variety of 16 sources, all in AEC license-exempt quantities. The standards are supplied in flame-sealed glass ampoules. They are ideal for making absolute calibrations of counting systems. The sources make easy-to-use relative sources for day-to-day calibration checks and come in various sizes and shapes to fit nearly all counting systems.

The set is also useful as a teaching aid for demonstrating calibration procedures for explaining nuclear phenomena such as absorption, back-scattering, and radiation characteristics.

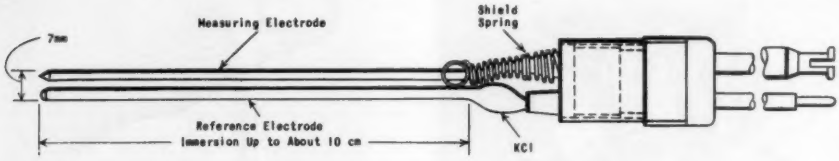
Miniature pH Electrode Assembly

A miniature pH electrode assembly is offered by Leeds & Northrup Company, for use with samples as small as one drop.

The two electrodes, reference and measuring, can be inserted in an opening 7 mm. in diameter, and a flexible electrode support allows the user to squeeze the two elements together and thus accommodate the single-drop sample, as in the tip of a centrifuge tube.

Use of the assembly is not limited to small samples; however, it may be used at any electrode immersion of 10 cm. or less. The pH range is 1.0 to 11.5; temperature range for samples is 15 to 40 C.

Self-shielding of the measuring electrode is said to eliminate the compartmental shielding usually required and avoids the need for sample transfer.



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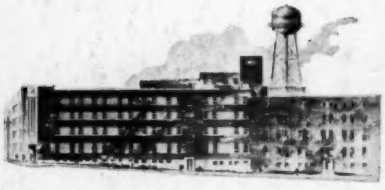
new research facilities for

PITMAN-MOORE COMPANY

Division of Allied Laboratories, Inc.

A $1,500,000 building, completed early in 1959, enables Pitman-Moore Company to concentrate in one location all types of research contributing to the development of human and veterinary medical preparations. This new unit adds 41,000 square feet of space for laboratories, offices, and technical equipment in the company's Research Center near Zionsville, Indiana.

The Indianapolis laboratories, where pharmaceuticals are produced.



The Zionsville laboratories, where biologicals are produced.

Within this organization originated the policy: Sales to Graduate Veterinarians ONLY

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